PHARMACODYNAMIC ANALYSIS OF *P. FALCIPARUM* KILL RATES IN MONOTHERAPY CLINICAL TRIALS AND INVESTIGATION OF QUORUM SENSING-DEPENDENCE OF EARLY KILL RATES IN MURINE MODELS

by
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Abstract

In treating malaria infections, rapid pharmacodynamic kill rates avert death and hinder drug resistance. We explored the recent 4 decades of published *P. falciparum* monotherapy drug trials featuring over 17,000 patients from 355 study arms from which we could extract pharmacodynamic data related to parasite reduction. Artemisinins represented 36%, methanolquinolines 27%, 4-aminoquinolines 20%, antifolates 10%, and antibiotics 7% of the useable trials. The fold-decrease in parasite count in 48 hours after treatment (PRR) and the time to clear 50% of the parasites (PC50) were robust metrics because either can be used to reliably predict the other. Other metrics like parasite clearance times (time to 90 or 100% clearance) or cure rate have clinical significance and are widely reported but provide little insight into pharmacodynamic rates. The critical first 48-hour PRR was around 5,100 for artesunate, 1,100 for quinine, 3,700 for chloroquine, 840 for sulfadoxine-pyrimethamine, 100 for atovaquone-proguanil, and 2 for clindamycin and azithromycin. After an extended lag, the antibiotics had a maximum PRR of 5,700, leading us to consider the time at which each drug reaches its maximum kill rate. All analyses were performed with the understanding that the trials had wide arrays of covariates such as initial parasitemia. In *Plasmodium*, physiological changes spurred by quorum sensing at high parasite density could affect drug sensitivity or parasite clearance. To better understand the effects of starting parasite density and quorum sensing on parasite kill rate, mouse models infected with *P. berghei* ANKA parasites expressing the GFP-luciferase reporter were treated with artesunate, amodiaquine, or pyronaridine at different times following infection. Mice were treated beginning at roughly 10,000, 100,000, or 1,000,000 parasites/μL (during log growth) or on day 6, 8, or 10 after infection (during the plateau phase). In subsequent arms, mice

were treated either before or after the projected “inflection point” between the log and plateau phases. In general, mice treated during the plateau cleared parasites slower immediately following treatment, but overall clearance and outcome was not significantly affected. This pattern was most exaggerated in artesunate, but there was also a notable distinction in mice treated with quinolines, especially at low doses.

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Chapter 1: Pharmacodynamic analysis of kill rates in P. falciparum monotherapy clinical trials

Introduction

Malaria remains a leading cause of morbity and mortality throughout much of the world, with over 200 million cases causing nearly half a million deaths in 2016. The most severe form of human malaria is caused by *Plasmodium falciparum*, which causes most of the malaria-related mortality seen in patients in southeast Asia and nearly all malaria mortality in Sub-Saharan Africa.

Most deaths from severe malaria occur within the first 24 to 48 hours of the onset of symptoms, so the prompt reduction of the parasite load within this window is essential. While numerous factors can play into the exact rate of parasite clearance, the drug administered is clearly the most important covariate affecting the rate and timing of the decline in parasitemia. As such, we set out to collect existing data in an attempt to corroborate or refute existing paradigms regarding the rate of parasite clearance characteristic of each drug.

This study, inspired largely by two of Dr. Nicholas White's reviews expounding the clinically-observed details of the malaria parasite clearance curve, consisted chiefly of compiling a large, if not exhaustive repository of clinical trial data and an initial analysis thereof. The discussion of this undertaking will be divided into three parts: the anatomy of the clinical trials, the aggregated results of these trials, and the ultimate utility (or futility) of such an endeavor.

For the current project, we chose to focus only on monotherapy trials. This could be a contentious decision, given that today, monotherapy is not recommended. However, trials featuring monotherapy were selected for several reasons. First, combination

therapies– especially experimental therapies that would be observed in clinical trials– contain multiple drugs that may be given in different ratios or schedules. This further complicates the plethora of covariates to be considered that will be discussed. Second, combinations of drugs may have effects on the kill rate that are not directly reflective of the constituent drugs; the partner drugs often enhace cure, but not clearance rate. We thought the additional data was not worth what we sacrifice in clarity regarding each individual drug. Finally, our lab uses murine models to study malaria drugs and regimens, and information regarding monotherapy will help to both ground these mouse studies in the human literature and inform future potential therapies to be tested in the mice by examining features of each drug that may be complementary.

The anatomy of the malaria monotherapy trial

Clinical trials, by their very nature, are interested primarily in the successes or failures of a treatment regiment. This principle introduces two confounding factors into the analysis of the body of clinical trials. The first, which is not specific to malaria, is the *sui generis* nature of every clinical trial. There is little impetus to perform many trials with the same covariates, so each trial is, more or less, an island. Features of the treatment– notably, the identity of the drug, its formulation and delivery method, the dose, the duration, and schedule– clearly affect the pharmacokinetics (PK) and/or pharmacodymanics (PD) in the patient and therefore have an undeniable effect on the outcome. Disease-specific factors like the severity of the disease or the species and strain of the infecting parasites may alter interactions with patients and with drugs. Demographic characteristics, such as sex, age, location, patient immune status, and the dates of the trial could have affects on the results by altering PK/PD as well; some covariates may alter the pharmacodynamic kill rate of a drug by mechanisms that are

neither recorded in these trials nor fully understood. Further, each study may use a different measure of central tendency and error term. In this light, it is with great trepidation that anyone pools data from clinical trials, as it is unclear to what extent the trials are fully comparable.

The second complication introduced by the central tenet of clinical trials as stated above is more specific to the treatment of malaria. The circulating burden of malaria, unlike that of many other systemic infections, can be quantified to a reasonable degree of accuracy. While this number does not represent the total body burden of total biomass because the percent sequestered at any point in time is not definable in *P.falcipuarm* infection, the estimate is nonetheless vital in that it is possible to observe differential rates of parasite clearance across different drugs *in vivo*. Despite rate of clearance being undoubtedly important to physicians because of its correlation with clinical outcome, the paramount concern will always be patient recovery and survival. As a result, many clinical trial reports– for reasons including, but not limited to, equipment or personnel restraints, financial concerns, or publication restrictions– forego either the collection or reporting of parasite clearance data and publish solely survival rates and other empirical results such as time to defervescence or coma resolution.

Nevertheless, many authors of clinical trial reports do choose to report some measure of parasite clearance rate. Clearance is usually defined by the average time to clear a predetermined percentage of parasites present at the beginning of treatment. These checkpoints are commonly 50% and 100% (or, more accurately, the time to absent *detectable* parasitemia), though 90%, 95% and 99% clearance times are also occasionally reported. The initial parasite count is also reported, because it is assumed that these

clearance times are to some degree dependent on this level (*i.e.,* clearance does not proceed in a first-order fashion). The parasite reduction ratio, the ratio of parasites at the onset of treatment to that after 1 full life cycle (48 hours for *P. falciparum*) is arguably the most well-known metric for describing parasite clearance rates, but it is rarely reported directly. Instead, it can be observed in a more robust descriptor of parasite clearance, which is the raw parasite count data depicted in an actual clearance curve.

The aggregated results of the trials to be obtained

By analyzing the available data from several clinical trials of malaria monotherapy, we hoped to compile a small database of results from which we could draw rough conclusions regarding the early kill rates of each drug. We attempted this by using some of the more commonly-reported metrics for parasite clearance, as well as devising new metrics that, while unrefined, could be used to describe empirically the rate of parasite clearance.

Parasite clearance time (time to absent detectable parasitemia) is perhaps the simplest metric with clinical significance. While it is commonly reported, it has limited utility in attempts to describe the clearance profile of any drug, because clearance time is necessarily related to initial parasitemia, with patients with lower initial parasite counts clearing their parasites earlier (White, 1997; Stepniewska *et al.*, 2010). The common geomean or average starting parasitemia also compresses the high and low initial parasitemias.

Parasite clearance is traditionally reported in a clinical setting as a clearance time or a parasite reduction ratio (PRR), but it must be acknowledged that a lag period following treatment is often observed, even to some degree in the fastest-acting drugs, artemisinin and its derivatives. This lag period confounds clearance measures because

any simple numerical descriptor of clearance rate will simply connect point A to point B without regard for what happens between them. The debate continues regarding whether this empirical approach is sufficient, as practitioners and patients alike remain dedicated to the reduction of the parasite load by any path necessary. On the contrary, antimalarials are often described (and, of course, marketed) by the maximum rate at which they clear parasites; this measure is considered less variable and more indicative of the mechanism of action of the drug (White 2011), but ignores important features of a drugs' PK/PD profile. In severe cases when patients may not survive to the end of the lag period, it is not sufficient to claim that a drug will work rapidly, but not immediately. The importance of the lag phase itself has been disputed. Once cited as a positive prognostic indicator due to the presumed synchronicity of the infection (Gashot *et al.*, 1996), an early rise in parasitemia following treatment is likely unrelated to outcome, though it is more common in complicated malaria (Silachamroon *et al.*, 2001).

Reconciling these two measures– clearance time and PRR– may prove impossible without complex modeling of each drug, which has been started elsewhere, but not undertaken here. Instead, we report both measures and will briefly consider the effects of each. As will be discussed in the next section, these results are subject to scrutiny and will be the subject of more intensive analysis in the future. For each of the results we find, there are dozens of correlations between covariates that we have yet to consider. The plethora of studies included in this study amounts to only a glimpse at the entire body of data freely available, but nonetheless the results are worthy of consideration.

The utility of the results

Due to the issues described above, we must tread carefully when considering any implications of the data we recover from these analyses. In addition, other studies may

throw into question the usefulness of studying the early kill rate of drugs at all. For example, it has long been assumed that the early clearance rate following treatment is that which would have occurred anyway, depending on the age distribution of the infecting parasites and whether schizogony or sequestration would predominate at that time (White, 1997). Khoury *et al* (2016) observed a significant positive correlation between the early parasite clearance rate following treatment and the growth rate 36 hours before treatment, even when parasite count decreased, indicating that kill rates are at least associated with, if not entirely dependent on, the growth cycle of the parasites before treatment. Hoshen and colleagues' (2000) model suggests that synchronicity is essential to the initial kill rate, and the more synchronous the infection, the faster the kill rate can be. Both of these results are significant variables that cannot be observed in our data or any other extracted dataset.

Immunity status may be the single largest factor affecting kill rate for any given drug, according to a multitude of studies in numerous locations (Lopera-Mesa *et al.,* 2013; Greenhouse *et al.,* 2009; Hastings *et al.,* 2015; White, 1997; Stepniewska *et al.,* 2010) and while it can be estimated based on endemicity of the region in which a patient lives, it cannot be quantified precisely for data pooled into a clinical trial. Geographical location is also an important factor in that it likely affects care-seeking behavior. Not only are more remote patients more likely to progress to severe malaria, patients near care facilities are more likely to receive treatment immediately following schizogony-induced fever (Mok *et al.,* 2014). Geographical distribution, parasite heterogeneity, patient age, and duration of study are factors that can affect immunity and treatment within any one trial, and these cofactors have yet to be accounted for in the present

analyses due to lack of data or an insufficient number of trials. Further, enhanced immune status could increase clearance rates by supplementing the drug's activity in either or both of the required actions for an observed reduction in parasite count– killing or splenic clearance of the parasites. Depending on the exact mechanism of the apparent drug-immune system synergy, immune status may impart a different effect on each drug. We point out that we are not conducting a meta-analysis.

In the absence of evidence to contrary, we will carry on under the assumption that pooling the data will provide the most reliable estimate of kill rates in spite of all the potential confounders. Our results will then be a nearly all-encompassing look at the various rate measures for each drug and will, at the very least, provide insight into the veracity of the assumptions in the field regarding the times, rates, and other characteristics of action for each drug. A network meta-analysis may provide a more succinct, statistically-relevant look at the data in the future.

Drug resistance was not considered a confounder in our analyses. First, parasite populations that are considered highly drug-resistant would not be treated with a monotherapy regimen of that drug. In addition, in the event of developing resistance, Hastings *et al* (2015) demonstrated that resistance has little impact on clearance rates unless sensitivity is extremely low.

The parasite clearance estimator (PCE) is a tool available from the Worldwide Antimalarial Resistance Network (WWARN) that uses an algorithm to predict an appropriate parasite clearance curve given important covariates (Flegg *et al.,* 2011). However, while repeated measures of parasitemia are required for input into the PCE (Jamsen *et al.,* 2013), we aimed to describe the clearance profiles of antimalarials using

the metrics commonly available in the literature. The PCE certainly represents a more comprehensive model based on only a few clinical trials than what we derived here, but its requirements are not often met in the field. Rather than producing a complicated model, we aimed to describe the features of the parasite clearance curve itself, as well as some trends through time and space, in simply numeric terms such that the entire profiles of the drugs could be compared.

Methods

Data Collection

Data were collected from 185 studies treating over 17,000 patients with monotherapy regimens. The dataset was compiled from searching databases for malaria monotherapy trials and from WWARN's register of malaria chemotherapy trials. PubMed and Google Scholar search terms included, but were not limited to, "malaria monotherapy trial", "*falciparum* chemotherapy", and searches for trials for specific malaria drugs. Then, the references of each selected paper were searched for relevant keywords. The current analysis was not strictly a meta-analysis or systematic review.

Inclusion Criteria

For a trial to be included in any analyses, it had to present at least one type of pharmacodynamic data: either a time at which a certain clearance threshold was met (commonly 50%, 90%, and absent parasitemia– PC50, PC90, and PCT respectively) or parasite counts at given times from which rates could be calculated. When parasite counts were the only source of clearance data, parasite counts must be provided at least once per day for the first 48 hours. GraphClick software was used to extract data points from graphs when PDFs of publications were available but the data itself was not provided.

Due to the vast number of reports available online via the Welch Medical Library, publications that were not available online were not included. Trials were also excluded if they featured no monotherapy treatment arms, infection with *P. vivax* (or mixed infections), or were not available in English. Two exceptions were made for combination therapies– sulfadoxine-pyrimethamine and atovaquone-proguanil– because they are used almost exclusively in combination with each other, and the constituent drugs are almost always combined in roughly the same ratio.

Metrics

Authors of malaria clinical trials report a wide array of metrics to describe the effectiveness of malaria chemotherapeutic agents. No metric for clearance rate was universal, which made comparison between trials difficult. Many trials do not report any clearance data, opting only to describe the effectiveness of the drugs with respect to cure or ablation of specific symptoms, such as coma or fever; such trials were excluded from the present analysis as per the inclusion criteria. The descriptors and metrics collected from each study are listed in Table 1.1.

METRIC	DESCRIPTOR	EQUATION
COUNTRY/REGION	Country and region from which data was collected	
INITIAL PARASITEMIA (P_0)	Patient parasitemia measure at t_0 (before treatment)	
ERROR	Error surrounding previous value; could be one number (SD) or bounds (CI, RANGE)	
CENTRAL TENDENCY TYPE	Reported measure of central tendency for previous value (e.g., MEAN, GEO. MEAN)	
ERROR TYPE	Reported error surrounding previous value (e.g., range, SD, 95% CI)	
DOSE ADMINISTERED	Amount of drug administered per dose; changes in dose throughout regimen denoted by "/"	
DOSES PER DAY	Number of doses given per day; "/" corresponds with changes in DOSE or represents loading dose	
DURATION OF TREATMENT	Length (in days) of treatment; "/" corresponds with changes in DOSE or FREQ	
FORMULATION/DELIVERY	Formulation of drug and/or route of delivery	
TOTAL DRUG GIVEN	Total amount of drug administered to a given time point	DOSE*FREQ*days
ABSOLUTE REDUTION RATE	Decrease in percentage of initial parasitemia (P_0)	(P1-P0)/[(t1-t0)*100]
PARASITES REMAINING	Raw number of parasites remaining	P(t)
PARASITES CLEARED	Raw number of parasites cleared (cumulative)	Abs.rate*p0 (+abs.rate1*p1, etc)
RELATIVE REDUCTION RATE	Parasitemia reduction ratio in 24 hour span	(P1/P0)/[(t1-t0)*100]
FOLD DECREASE	Inverse relative refuction rate; fold decrease in 24 hour span	1/(rel.rate)
PARASITE REDUCTION RATIO	Ratio of parasitemia at onset of treatment to parasitemia at 48 hours	P0/p2
PC1/2 (h)	Calculated time to clear half of remaining parasites (based on decrease in 24 hour period)	24/log2(fold.decrease)
	More robust calculation of PC1/2 accounting for paradoxical increases; number becomes more arbitrary but can be better displayed	24/log2(fold.decrease), fold.decrease>1.05; 24/log2(fold.decrease)-24/log2(0.95)4/log2(1.05), fold.decrease≤0.95

LOG RELATIVE REDUCTION RATE	Log transformation of fold decrease	Log(fold.decrease)
LOG REDUCTION (0-48)	Log reduction in initial parasitemia in 1 life cycle (0-48 h)	Log(p0-p2)
EST. PARASITES CLEARED	Estimate of parasites cleared in 48 hours based on PC50 as a half-life	P0-p0/(48/pc50)
EST. PARASITE REDUCTION RATIO	PRR calculated based on PAR RED*	P0/par.red*
EST. PARASITES CLEARED	Estimate of parasites cleared in 48 hours based on PC1/2 as a half-life	P0-p0/(48/pc1/2)
PC50	Measured time to clear 50% of initial parasitemia	
EST. PC50	Interpolation of PC50 from clearance data	y=50%
EST. PARASITES CLEARED	Estimate of parasites cleared in 48 hours based on PC50* as a half-life	P0-P0/(48/PC50*)
PC90	Measured time to clear 90% of initial parasitemia	
PCT	Measured time to absent measurable parasitemia	
NUMBER OF PATIENTS	Number of people treated	
CURE RATE	Percentage of patients who remain clear of parasitemia (and symptoms) at end of follow up	
SURVIVAL RATE	Percentage of patients who survive to end of study	1-MORTALITY
RECRUDESCENCE	Percentage of patients who recrudesce	
FOLLOW UP TIME	Follow up time	
MINIMUM PC1/2	Minimum PC1/2 throughout treatment	iterative calculations– 24/LOG2(P(N)/P(N+1))
MiNIMUM PC1/2 TIME	Time at which minimum PC1/2 is reached	
MAXMUM PRR	Maximum PRR thoughout treatment	iterative calculations– P(N)/P(N+2)
MAXIMUM PRR TIME	Time at which maximum PRR is reached	
T_{LAG}	Estimate of lag time before drug is (maximally) effective	iterative slopes– $(P_{n+1}-P_n)/(t_{n+1}-t_n)=S$; considered t_{lag} if $S_n/S_{max}<0.2$ (before $S_n/S_{max}>0.2$ for 2 time points)
ADJUSTED PC1/2	PC1/2 adjusted to exclude lag phase	
ADJUSTED PRR	PRR adjusted to exclude lag phase	

Table 1.1 *Name, description, and formula (when applicable) for metrics used in analyzing* P. falciparum *monotherapy clinical trial data*

Lag time was defined according to the PCE algorithm as the time until the log-adjusted slope of the parasite clearance curve was greater than 20% of the maximum slope throughout observation (Flegg *et al.,* 2011). We added the provision that the slope must remain at or above 20% of the maximum slope for at least two consecutive data points. Identification of the maximum slope was constrained such that the first point must occur when the parasite count is at least 10% of the initial count in order to avoid large slopes owing only to imprecision at such low parasite levels.

Some other metrics, notably "$PC_{1/2}$", were devised specifically for this analysis to make data for each trial reporting clearance data more robust and comparable. Finally, in order to ascertain the "ideal" effectiveness of a particular drug irrespective of its lag time (which may hinder its effectiveness in practice), "adjusted" and maximum PRR and PC ½ were calculated to demonstrate rate excluding the lag period and the fastest rate throughout the regimen, respectively.

$PC_{1/2}$ was devised as a proxy for the commonly-reported PC50 for use in trials where PC50 is not available. It operates under the assumption that, for early time points following treatment, parasite count follows a first-order decay pattern, wherein PC50 becomes comparable to a half-life; such has been observed to be at least roughly true (Lopera-Mesa *et al.,* 2013; Day *et al.,* 1996). $PC_{1/2}$ can be calculated according to the following equation:

$$PC_{1/2} = \begin{cases} \dfrac{24}{\log_2 \frac{P_1}{P_0}}, & \dfrac{P_1}{P_0} > 1.05 \\ undef., & 0.95 < \dfrac{P_1}{P_0} < 1.05 \\ \dfrac{-24}{\log_2 \frac{P_1}{P_0}} + \dfrac{24}{\log_2 1.05} + \dfrac{24}{\log_2 0.95}, & \dfrac{P_1}{P_0} < 0.95 \end{cases}$$

Equation 1.1 *$PC_{1/2}$*

$PC_{1/2}$ is undefined when there has been a change in parasite count of less than 5% because of the large error that could result from extrapolation from such a small sample. In situations where the parasite count increases following treatment, the $PC_{1/2}$ that would be calculated from the traditional formula is negative, which, if trials with paradoxical increases in parasite density are included in analyses, will artificially deflate the pooled $PC_{1/2}$, creating the illusion of faster clearance. As such, the opposite of the $PC_{1/2}$ from the traditional equation is added to a baseline value representing the maximum $PC_{1/2}$ value before the result becomes undefined. In this way, a graph of $PC_{1/2}$ vs. fold decrease in parasite count will appear continuous, save the small region wherein $PC_{1/2}$ is undefined, and paradoxical increases are more accurately represented as slow clearances.

P_{red}* is calculated according to the same principle likening parasite clearance to radioactive decay. In this case, when data describing parasite clearance through time are unavailable, the number of parasites eliminated was estimated from PC50 by the following equation:

$$P_{red}{}^{*} = P_0 - \frac{P_0}{2^{\frac{48}{PC50}}}$$

Equation 1.2 $P_{red}{}^{*}$

Where 48/PC50 signifies the number of half-lives that occur in 48 hours, so $(1/2)^{48/PC50}$ represents the fold decrease as a result of this level of "decay"; multiplication of this value by the initial parasite count should result in the number of parasites remaining after 48 hours. P_{red}* could then be used in place of parasite count at the later time point when calculating PRR.

Analyses

Data for the various analyses were collected in the Prism GraphPad program, which performed the necessary calculations for each (GraphPad Prism version 7.0b for Mac). Among the simple analyses we performed were comparisons of PRR, PC50, and percent of parasites remaining through time. $PC_{1/2}$ was calculated as a proxy for PC50 in trial arms that provided clearance data, while P_{red}* was calculated as an estimate of the number of parasites reduced over 48 hours given PC50. Finally, analyses considering the lag phase involved consideration of both the magnitude and time of the maximum parasite clearance rate (as measured by $PC_{1/2}$ or PRR). Each analysis was conducted within specific drugs and within drug classes.

One specific analysis that was performed was to look for a relationship between initial parasitemia and kill rate (PRR). Initial parasite count was stratified into *low* ($P_0=10^{3.0\pm0.5}$), *medium* ($P_0=10^{4.0\pm0.5}$), *high* ($P_0=10^{5.0\pm0.5}$), and *high*+ ($P_0>10^{5.5}$). When data were available, PRRs for trials within each stratum were plotted. Another visualization was created showing the distribution of times at which the kill rate was highest in each trial. This allows comparison of drugs beyond raw rates, but instead contextualizes them, accounting for lag phases.

Statistical analyses have yet to be reliably performed for any analysis. Just as the metrics reported vary greatly, so too do the error terms reported and the measures of central tendency. It should be possible to approximately convert between these measures using some combination of formulae and bootstrapping methods, but we chose instead to simply treat each study arm as an individual mean, with an associated weight according to the number of participants, and use the standard deviation of these weighted means as the error terms in our analyses. We feel this provides at least enough evidence for a

qualitative assessment of commonly held beliefs regarding the kill rate associated with malaria drugs and drug classes. All analyses were performed with both weighted an unweighted means unless otherwise noted.

Results

Results of literature search

Our rudimentary literature search returned 164 trials with at least one study arm that satisfied the inclusion criteria. Within these 164 trials, there were 355 monotherapy study arms and 17553 patients observed. The drugs considered for analysis fell into 6 groups, with atovaquone-proguanil (and, occasionally, each drug by itself) occupying its own, seventh group. The classes, drugs, and abbreviations used for the drugs are delineated in Table 1.2.

CLASS	DRUGS (AND ABBREVIATIONS)
Aminoquinolines	Amodiaquine (AQ), Chloroquine (CQ), Pyronaridine (PYN)
Antibiotics	Azithromycin (AZ), Clindamycin (CL), Fosmidomycin (FOS),
Antifolates	Sulfadoxine-Pyrimethamine (SP)
Artemisinins	Artemisinin (ART), Artemether (AM), Artesunate (AS)
Methanolquinolines	Mefloquine (MQ), Quinine (QN)
Other	Atovaquone-Proguanil (AV+PG)
Other Hemozoin inhibitors	Halofantrine (HL)

Table 1.2 *Drugs featured in analyses of monotherapy trials. Abbreviations from WWARN.*

Of the 355 study groups used in at least one of our analyses, 49.9% (n=177) were from Southeast Asia and 47.0% (n=167) were from Africa. The remaining studies were from South America (2.5%, n=10), listed no location (n=1), or were composed of travelers returning from both Southeast Asia and Africa (n=1). Artemisinins were the most common drugs in these trials, composing 34.7% (n=123) of all study arms. Aminoquinolines composed 20.8% (n=75), antibiotics 4.8% (n=17), antifolates 8.5%

(n=30), methanolquinolines 23.7% (n=85), other hemozoin inhibitors 3.7% (n=14), and AV+PG or its substituent drugs 3.7% (n=14) of the remaining trial arms.

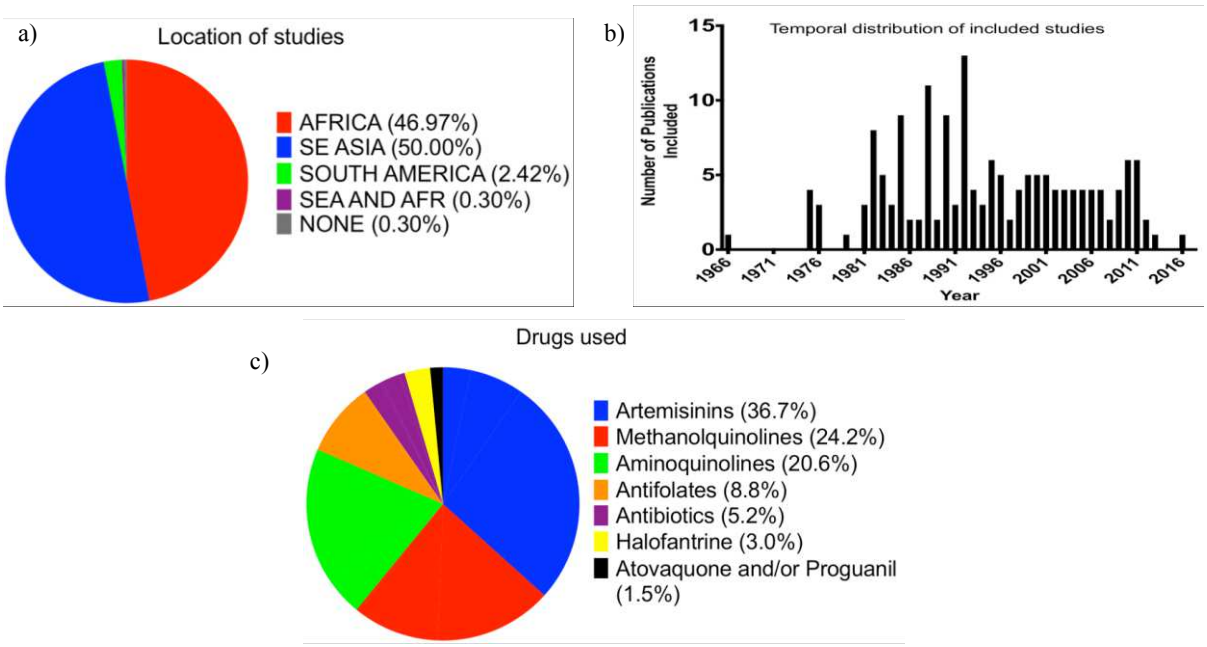

Figure 1.1 *Characteristics of studies included in the analysis. a) Geographical distribution of monotherapy study arms, b) dates of publication of all reports contributing at least one study arm to the analyses, and c) frequency of each drug class having been used in a monotherapy study arm*

The drug regimens we encountered are described in Table 1.3. To avoid decreasing sample sizes by stratification, we were forced to adopt the assumption that all doses, durations, and schedules of drug administration provide similar kill rates. While this may not be strictly true, it can be justified to some degree in that patients are unlikely to be given a dose low enough so that the available drug is the rate-limiting component.

DRUG	FIRST DOSE (MG/KG)	DURATION (DAYS)	MULTIPLES OF FIRST DOSE PER DAY	PATIENTS TREATED
Artemesinin	1.67	5	2, 2, 5, 5, 5	37
	8.3	6	1, 1, 1, 1 ,1 ,1	30
	8.33	5	1, 1, 1, 1, 1	40
	10	2	2, 1.33	18
		3	1, 0.5, 0.5	10
			2, 1.33, 1.33	32
	12	3	2, 1.25, 1	30
	16.67	2	1, 1	10
		3	1, 1, 1	20
	40	4	1.33, 0.5, 0.5, 0.5	37
Artemether	1.6	6	2, 1, 1, 1, 1, 1	23
	2.67	5	1, 0.5, 0.5, 0.5, 0.5	86
		7	1, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5	15
	3.2	3	1, 0.5, 0.5	83
		4	1, 0.5, 0.5, 0.5	308
		5	1, 0.5, 0.5, 0.5, 0.5	193
	3.33	2	2, 1	31
		4	1.5, 0.5, 0.5, 0.5	34
		5	1, 0.5, 0.5, 0.5, 0.5	81
	4	7	1, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5	60
	5	5	1, 0.33, 0.33, 0.33, 0.33	13
	10.67	7	3, 3, 3, 3, 3, 3, 3	14
Artesunate	1	3	2, 2, 1	31
	1.6	5	1.5, 1, 1, 1, 1	25
	1.67	5	1.5, 0.5, 0.5, 0.5, 0.5	40
			1.5, 1, 1, 1, 1	25
			2, 1, 1, 1, 1	25
		6	1, 2, 2, 2, 2, 2	20
	2	7	1, 1, 1, 1, 1, 1, 1	187
	2.4	6	1.5, 1, 1, 1, 1, 1	40
	3	4	1, 0.67, 0.67, 0.67	37
	3.33	3	1, 1, 1	68
	4	3	1, 1, 1	30
		7	1, 1, 1, 1, 1, 1, 1	120
	6	7	1, 1, 1, 1, 1, 1, 1	51
	6.67	5	1, 0.5, 0.5, 0.5, 0.5	45
		7	1, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5	46
	10	1	1	114
Mefloquine	5.4	1	1	12
	8.33	1	1	90
	10.4	1	1	15
	12.5	1	1	155
		1	1.33	50
		1	1.67	103
	15	1	1	40
	16.67	1	1	162
	20	1	1	47
	20.83	1	1	215
	21.2	1	1	8
	25	1	1	664
	30	1	1	42
Quinidine	10	7	3, 3, 3, 3, 3, 3, 3	33
Quinine	8	3	3, 3, 3	22
	8.3	7	3, 3, 3	22
	8.33	3	3, 3, 3	10
		7	3, 3, 3, 3, 3, 3, 3	30
		14	3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3	24

	9	3	3, 3, 3	3
		7	3, 3, 3, 3, 3, 3, 3	11
	10	1	3	30
		5	3, 3, 3, 3, 3	21
		7	3, 3, 3, 3, 3, 3, 3	360
		8	3, 3, 3, 3, 4.5, 4.5, 4.5, 4.5	33
	10.8	10	3, 3, 3, 3, 3, 3, 3, 3, 3, 3	58
	12	7	3, 3, 3, 3, 3, 3, 3	12
		2	2, 1	37
		3	2, 2, 2	31
	16	7	2, 1.5, 1.5, 1.5, 1.5, 1.5, 1.5	50
	20	5	1.5, 0.5, 0.5, 0.5, 0.5	288
		7	2, 1.5, 1.5, 1.5, 1.5, 1.5, 1.5	394
Halofantrine	1	1	3	12
	4.17	1	3	28
	8	1	2	41
			3	145
			4	187
	8.33	1	3	112
	10	1	2	42
	16.67	1	1.5	20
	25	1	1	50
Pyrimethamine	1.25	1	1	24
Sulfadoxine+Pyrimethamine	(16.67)+(0.83)	1	1	52
	(25)+(1.25)	1	1	1094
		2	1.67, 0.67	21
	(25)+(1.5)	1	1	40
	(35)+(1.75)	1	1	28
	(35)+(2)	1	1	44
Amodiaquine	6.67	4	2, 1, 1, 1	24
	10	2	1, 0.5	22
			1.67, 0.67	47
		3	1, 0.75, 0.75	25
			1, 1, 0.5	185
			1, 1, 1	182
			1.5, 0.5, 0.5	17
		4	1, 0.5, 0.5, 0.5	14
	15	3	1, 0.67, 0.67	84
Chloroquine	5	1	1	24
	10	1	1	252
			5	48
		2	2, 1	19
		3	1, 1, 0.5	1312
			1, 1, 1	106
			1.5, 0.5, 0.5	91
	15	3	1, 0.67, 0.67	39
			1, 1, 1	41
	16.67	3	1.5, 1.5, 0.5	18
	16.7	3	1, 1, 1	16
	20	3	1, 1, 0.5	66
Pyronaridine	5	5	2, 1, 1, 1, 1	101
	8	3	2, 1, 1	81
Atovaquone	12.5	2	3, 1	25
		7	3, 3, 3, 3, 3, 3, 3	23
Atovaquone+Proguanil	(8.33)+(3.33)	2	2, 1	24
		3	2, 2, 2	30
		5	2, 2, 2, 2, 2	24
	(12.5)+(4.17)	(2)+(7)	(3, 1)+(1, 1, 1, 1, 1, 1, 1)	25
	(12.5)+(5)	3	1, 1, 1	55

	(16.67)+(6.67)	3	1, 1, 1	196
	(17)+(7)	3	1, 1, 1	30
	(20)+(8)	3	1, 1, 1	92
Proguanil	8.33	3	2, 2, 2	13
Azithromycin	16.7	3	1, 1, 1	16
Clindamycin	5	5	1, 1, 1, 1, 1	44
			2, 2, 2, 2, 2	111
	7.5	3	3, 3, 3	12
	10	5	2, 2, 2, 2, 2	12
Fosmidomycin	20	3	3, 3, 3	10
		4	3, 3, 3, 3	8
		5	3, 3, 3, 3, 3	9
		7	3, 3, 3, 3, 3, 3, 3	35
	30	5	2, 2, 2, 2, 2	24

Table 1.3 *Description of all monotherapy treatment regimens present in included trial reports (where sufficient data were available; 74 arms lacked duration of treatment– mostly those recovered from WWARN Parasite Clearance Study Group's meta-analysis [2015]– and one lacked a sample size).*

The variables reported in the included trials are shown in Table 1.4. Because the metrics given were a significant component of the inclusion criteria for the analyses, the values shown are not representative of the entire body of malaria chemotherapy literature. Instead, they are only a description of the metrics reported provided that at least one clearance measure was given.

		ARTEMISININ		ARTEMETHER		ARTESUNATE		MEFLOQUINE		QUINIDINE		QUININE	
Clearance Measures	Initial parasite count	11	100.00%	20	95.24%	88	96.70%	35	92.11%	1	100.00%	40	90.91%
		n=254	100.00%	n=969	77.09%	n=6458	97.98%	n=1535	93.60%	n=33	100.00%	n=1571	80.19%
	Parasitemia measurements	5	45.45%	9	42.86%	7	7.69%	12	31.58%	0	0.00%	15	34.09%
		n=80	31.50%	n=549	43.68%	n=319	4.84%	n=478	29.15%	n=0	0.00%	n=666	34.00%
	PC50	8	72.73%	7	33.33%	82	90.11%	2	5.26%	0	0.00%	16	36.36%
		n=214	84.25%	n=540	42.96%	n=6249	94.81%	n=315	19.21%	n=0	0.00%	n=1196	61.05%
	PC90	3	27.27%	5	23.81%	79	86.81%	2	5.26%	0	0.00%	12	27.27%
		n=85	33.46%	n=503	40.02%	n=6179	93.75%	n=315	19.21%	n=0	0.00%	n=1137	58.04%
	PCT	11	100.00%	20	95.24%	25	27.47%	32	84.21%	1	100.00%	40	90.91%
		n=254	100.00%	n=969	77.09%	n=951	14.43%	n=1190	72.56%	n=33	100.00%	n=1889	96.43%
Clinical Measures	Cure rate	5	45.45%	10	47.62%	25	27.47%	32	84.21%	0	0.00%	21	47.73%
		n=119	46.85%	n=336	26.73%	n=1105	16.77%	n=1065	64.94%	n=0	0.00%	n=740	37.77%
	Recrudescence (RI) rate	8	72.73%	12	57.14%	23	25.27%	30	78.95%	1	100.00%	23	52.27%
		n=216	85.04%	n=384	30.55%	n=869	13.18%	n=1000	60.98%	n=33	100.00%	n=775	39.56%
	Total	11		21		91		38		1		44	
		n=254		n=1257		n=6591		n=1640		n=33		n=1959	
		HALOFANTRINE		**PYRIMETHAMINE**		**SP**		**AMODIAQUINE**		**CHLOROQUINE**		**PYRONARIDINE**	
Clearance Measures	Initial parasite count	10	71.43%	1	100.00%	26	89.66%	14	93.33%	44	84.62%	4	100.00%
		n=453	71.11%	n=24	100.00%	n=1089	84.55%	n=531	88.50%	n=1621	154.82%	n=182	100.00%
	Parasitemia measurements	6	42.86%	0	0.00%	8	27.59%	5	33.33%	30	57.69%	2	50.00%
		n=336	52.75%	n=0	0.00%	n=261	20.26%	n=162	27.00%	n=1118	106.78%	n=101	55.49%
	PC50	2	14.29%	0	0.00%	0	0.00%	0	0.00%	2	3.85%	2	50.00%
		n=160	25.12%	n=0	0.00%	n=0	0.00%	n=0	0.00%	n=34	3.25%	n=101	55.49%
	PC90	2	14.29%	0	0.00%	0	0.00%	0	0.00%	1	1.92%	0	0.00%
		n=160	25.12%	n=0	0.00%	n=0	0.00%	n=0	0.00%	n=15	1.43%	n=0	0.00%
	PCT	11	78.57%	1	100.00%	23	79.31%	13	86.67%	31	59.62%	4	100.00%
		n=398	62.48%	n=24	100.00%	n=1049	81.44%	n=516	86.00%	n=1198	114.42%	n=182	100.00%
Clinical Measures	Cure rate	11	78.57%	1	100.00%	25	86.21%	15	100.00%	30	57.69%	4	100.00%
		n=427	67.03%	n=24	100.00%	n=966	75.00%	n=600	100.00%	n=1340	127.98%	n=182	100.00%
	Recrudescence (RI) rate	8	57.14%	1	100.00%	21	72.41%	13	86.67%	25	48.08%	4	100.00%
		n=391	61.38%	n=24	100.00%	n=763	59.24%	n=496	82.67%	n=1097	104.78%	n=182	100.00%
	Total	14		1		29		15		52		4	
		n=637		n=24		n=1288		n=600		n=1047		n=182	

		ATOVAQUONE		ATOVAQUONE+ PROGUANIL		PROGUANIL		AZITHROMYCIN		CLINDAMYCIN		FOSMIDOMYCIN	
Clearance Measures	Initial parasite count	2	100.00%	9	90.00%	1	100.00%	1	100.00%	7	100.00%	7	100.00%
		n=48	100.00%	n=421	88.45%	n=13	100.00%	n=16	100.00%	n=179	100.00%	n=86	100.00%
	Parasitemia measurements	0	0.00%	3	30.00%	0	0.00%	1	100.00%	5	71.43%	1	14.29%
		n=0	0.00%	n=176	36.97%	n=0	0.00%	n=16	100.00%	n=155	86.59%	n=12	13.95%
	PC50	0	0.00%	1	10.00%	0	0.00%	0	0.00%	0	0.00%	1	14.29%
		n=0	0.00%	n=30	6.30%	n=0	0.00%	n=0	0.00%	n=0	0.00%	n=20	23.26%
	PC90	0	0.00%	1	10.00%	0	0.00%	0	0.00%	0	0.00%	1	14.29%
		n=0	0.00%	n=30	6.30%	n=0	0.00%	n=0	0.00%	n=0	0.00%	n=20	23.26%
	PCT	2	100.00%	10	100.00%	1	100.00%	0	0.00%	3	42.86%	6	85.71%
		n=48	100.00%	n=476	100.00%	n=13	100.00%	n=0	0.00%	n=50	27.93%	n=74	86.05%
Clinical Measures	Cure rate	2	100.00%	10	100.00%	1	100.00%	1	100.00%	4	57.14%	6	85.71%
		n=48	100.00%	n=476	100.00%	n=13	100.00%	n=16	100.00%	n=97	54.19%	n=74	86.05%
	Recrudescence (RI) rate	0	0.00%	4	40.00%	0	0.00%	1	100.00%	4	57.14%	3	42.86%
		n=0	0.00%	n=257	53.99%	n=0	0.00%	n=16	100.00%	n=97	54.19%	n=47	54.65%
	Total	2		10		1		1		7		7	
		n=48		n=476		n=13		n=16		n=179		n=86	

Table 1.4 *Metrics reported according to treatment. Total number of study arms and total patients treated (n) are given for each drug/metric combination; percentages are percent of total study arms or total patients treated for a given drug.*

Parasite Clearance Time

PCT, as has been discussed previously, is one of the less robust clearance measures. Nevertheless, it is the most common clearance metric reported in the literature, so we report the PCT for each drug group in Figure 1.2. Artemisinins were, by far, the fastest to clear all detectable parasites. Antibiotics, such as clindamycin and fosmidomycin, were the slowest group; however, all other drug groups were fairly close with regard to PCT.

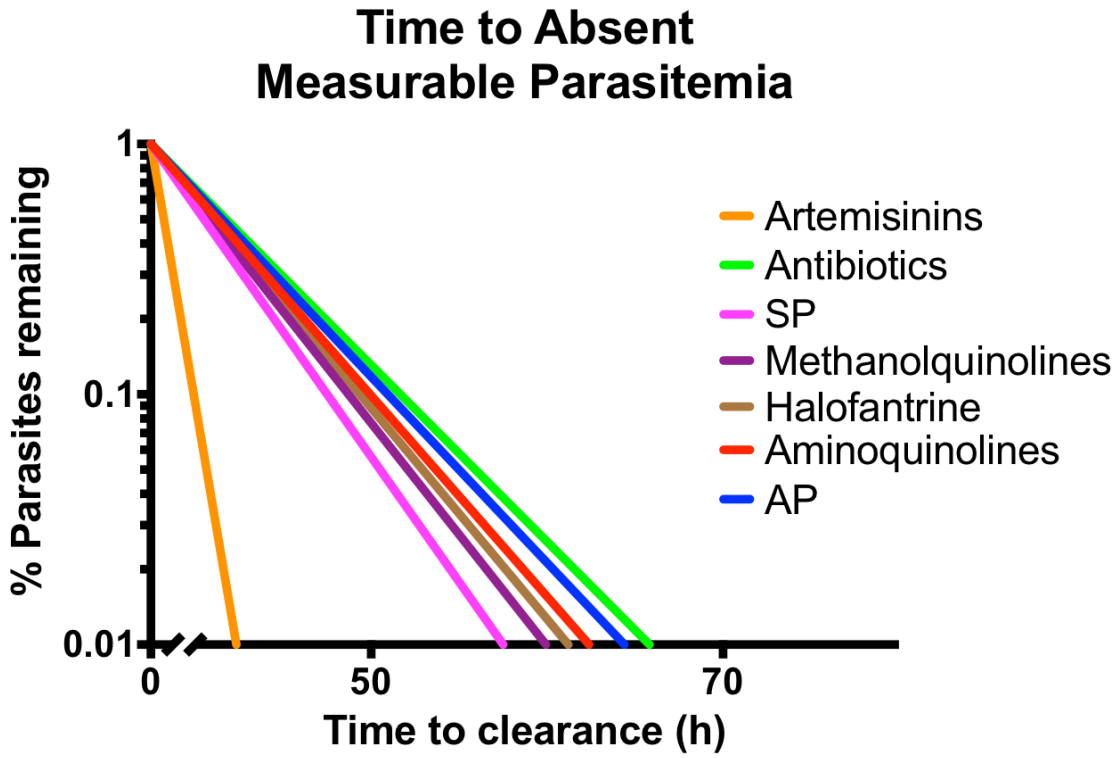

Figure 1.2 *Time to absent detectable parasitemia, based on weighted means in each drug group*

Parasite reduction ratio

Looking at the classes as a whole, the artemisinins were the fastest killing drug class with a PRR of over 10000, though artemether was notably faster than the other drugs in the class. Methanolquinolines and halofantrine had nearly the same PRR, with the aminoquinolines only slightly slower. Antifolates, atovaquone-proguanil, and

antibiotics were the slowest (Figure 1.3). It should be noted that the error terms shown are simply a standard deviation of the weighted means, as no error terms were available for most of the data from which our values were calculated. As such, we were forced to treat each result as one (weighted) mean and report the error based on the variability of those means.

No significant differences in PRR were observed when the same analysis was performed without regard for the size of the studies. This unweighted analysis was used to perform a D'Agostino & Pearson normality test to check if the PRR values obtained within each drug were log-Normally distributed. Of the drugs with a sufficiently large number of trials (AM, AS, MQ, QN, SP, and CQ), only CQ deviated significantly from a log-Normal distribution (α=0.05). A ROUT test to identify outliers (Q=1%) identified one CQ study as an outlier; excepting this study, the PRRs associated with CQ are log-Normally distributed.

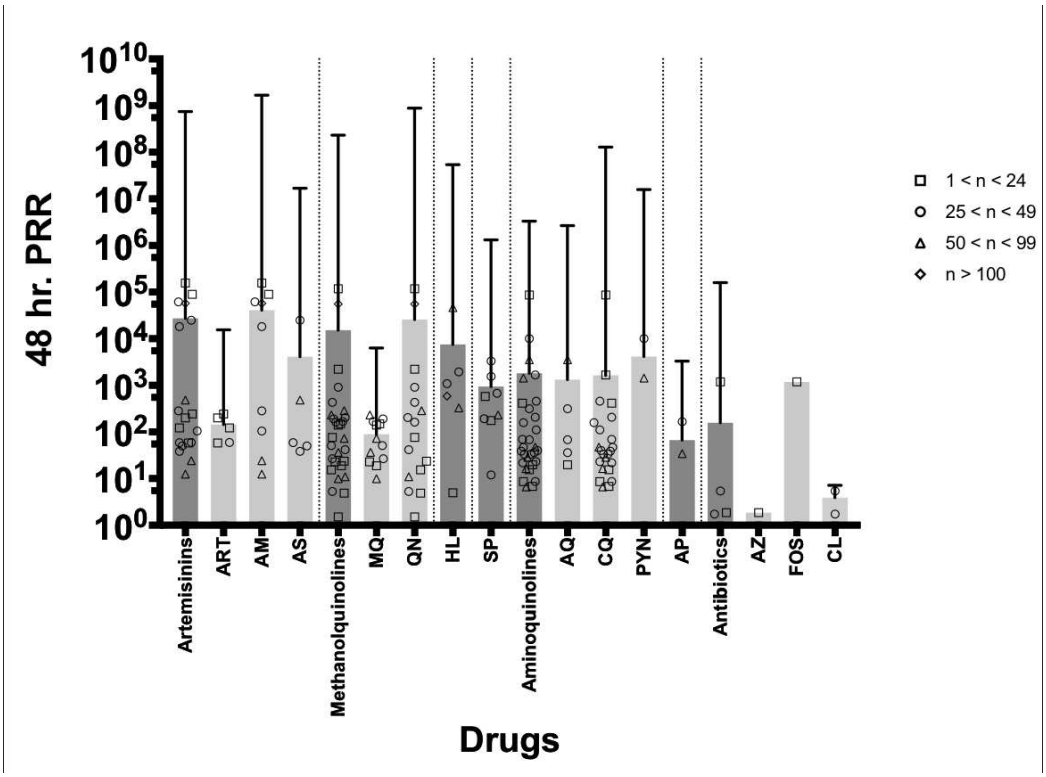

Figure 1.3 *PRR results for each drug and drug class. Vertical lines separate the drug classes. Each point represents one study arm, with its shape signifying the size of the arm.*

The results following stratification according to initial parasitemia were confounding. There was no universal trend wherein a higher initial parasite count was associated with faster or slower clearance. In some cases, there was no difference between strata (such as ART); in others, higher parasitemias tended to yield higher PRRs (e.g., CQ); in yet others, higher parasitemias were associated with lower kill rates (e.g., AS). In order to reduce the effect of multiple doses on PRR, while also reducing the impact of the lag phase, we chose to calculate a 24-hour PRR. These data are presented in Figure 1.4a. Whereas PRR is traditionally calculated across the first 48-hour life cycle following initiation of treatment, we thought it may also be informative to consider maximum PRR, calculated across whichever 48-hour span featured the largest fold-decrease in parasite count. When looking at the data this way, many of the trends within

a given drug are the same, but PRR still failed to correlate with starting parasite count in any systematic way (Figure 1.4).

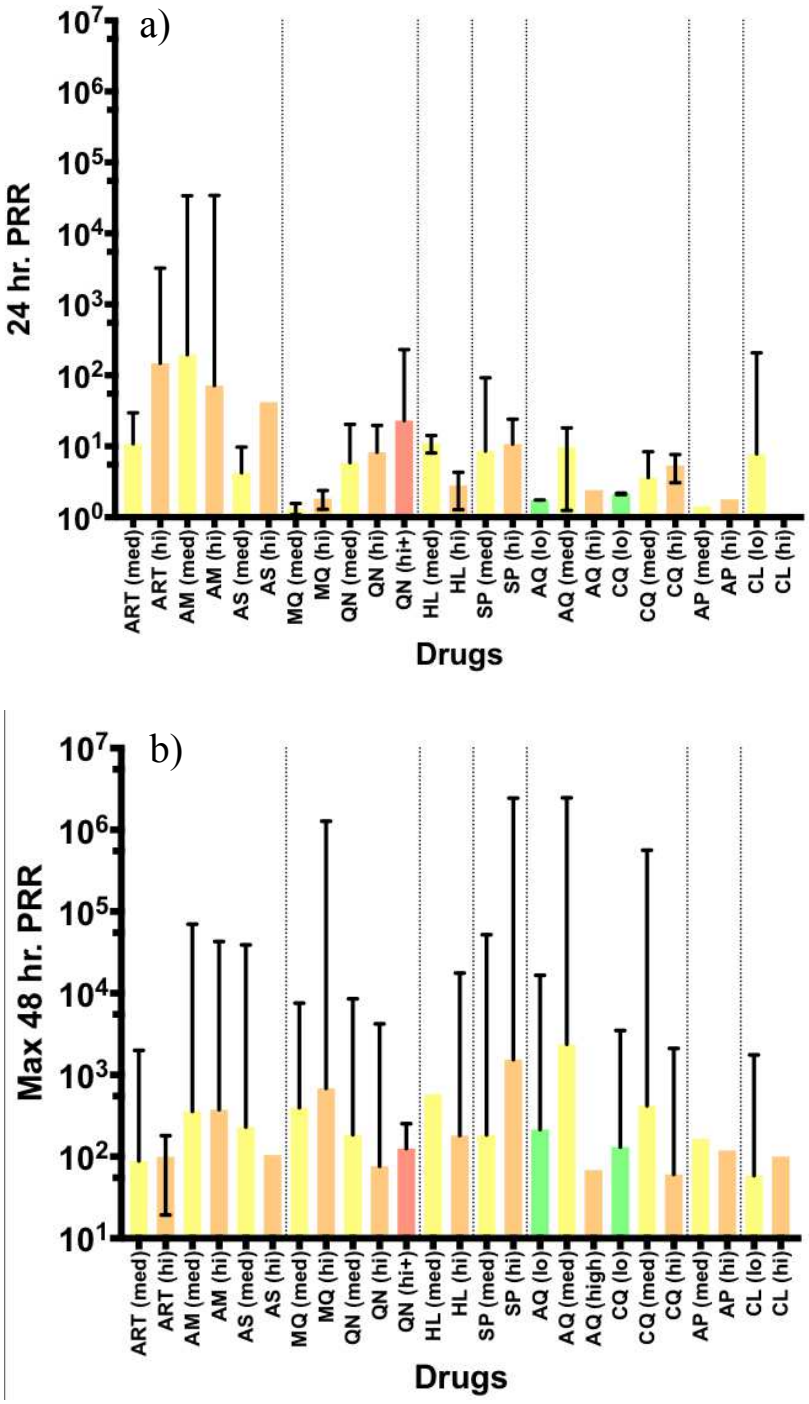

Figure 1.4 *PRR, stratified by initial parasite count. Strata are centered around 1000 (lo), 10000 (med), 100000 (hi), and 1000000 (hi+) parasites/μL (on a logarithmic scale). Drugs were excluded if all available trials fell into one stratum (PYN, AZ, FOS). a) initial 24-hour PRR, b) maximum PRR across any 48 hour span.*

Evaluation of new metrics

Neither $PC_{1/2}$ nor P_{red}* were considered reliable substitutes for PC50 or the number of parasites cleared, respectively. $PC_{1/2}$ was highly accurate in a select few cases, but in general, when comparing trials with sufficient data to compare $PC_{1/2}$ and PC50, $PC_{1/2}$ was off the mark in a non-systematic way. Future attempts to mathematize parasite clearance in this way may need to make adjustments for covariates that, for this preliminary attempt, were ignored. For the remainder of this study, where $PC_{1/2}$ is used, it is only used for its robustness in describing parasite clearance curves compared to PRR, rather than as a replacement for PC50.

P_{red}* was far more accurate than was $PC_{1/2}$ when compared to studies with both PC50 and clearance data available; almost all of the studies exhibited less than 10% error, while none exceeded 18% error. While these discrepancies are smaller than those seen when assessing $PC_{1/2}$, they do not instill confidence that PC50 can reliably allow the calculation of parasites cleared in every case (Figure 1.5).

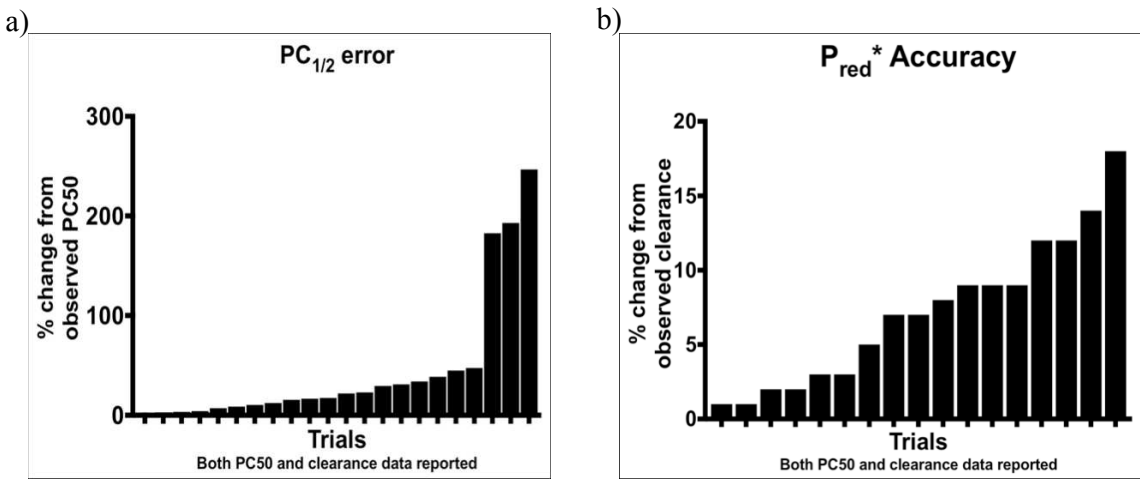

Figure 1.5 *Percent error of a) PC1/2 and b) Pred*, where the calculated value (PC1/2 and Pred*) is the observed value and the value derived from the reported data (PC50 and the number of parasites cleared) is the theoretical value. All trials where PC50 and clearance data were available were included; no correlation between percent error and drug or drug class was found.*

When we considered $PC_{1/2}$ on days 1, 2, and 3, most drug classes seem to exhibit higher and more variable $PC_{1/2}$ values on day 1 than on subsequent days following treatment (Figure 1.6). This is largely reflective of the lag phase, which may delay clearance for all or part of the first day following treatment. Therefore, fast-acting drugs like the artemisinins had $PC_{1/2}$ values on day 1 that were in better accordance with later

days.

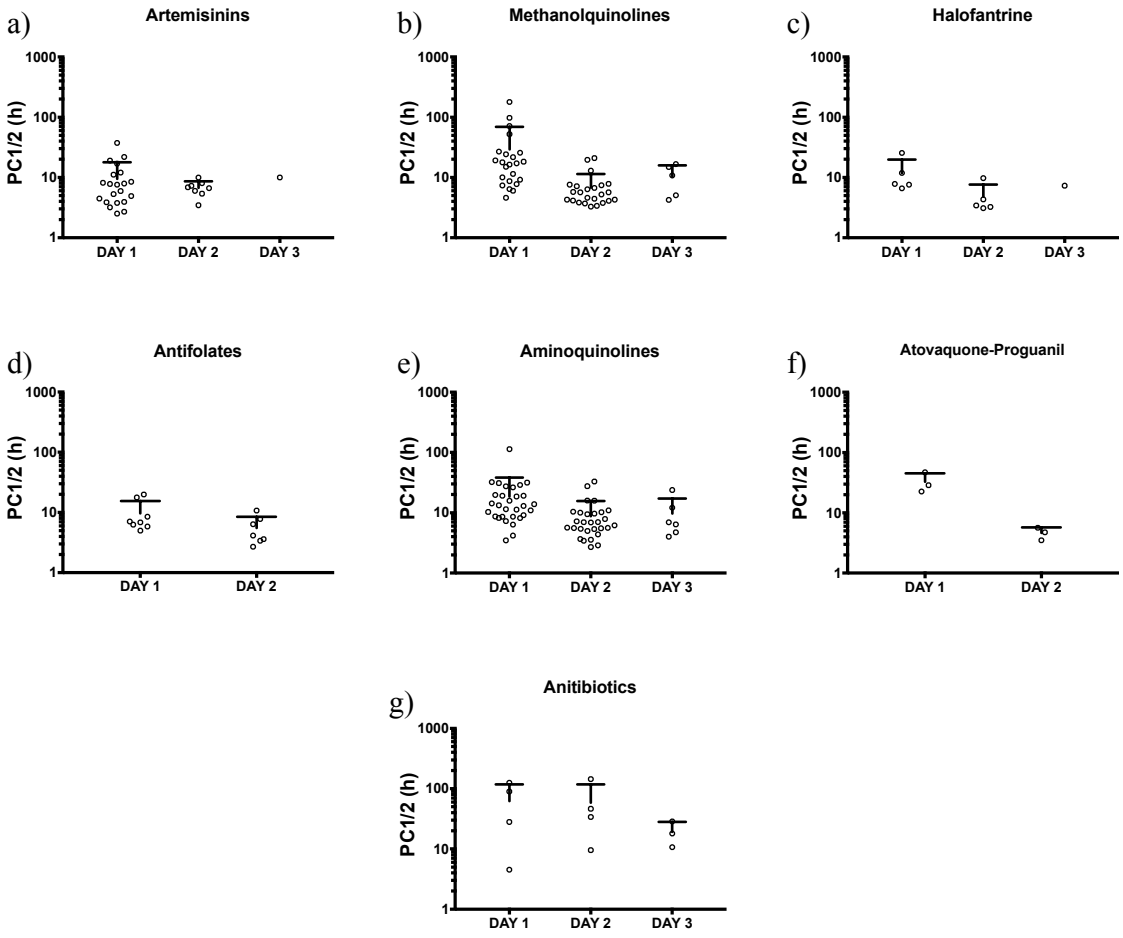

Figure 1.6 *PC1/2 for each drug class based on clearance during day 1 (0-24 hours), day 2 (24-48 hours), and day 3 (48-72 hours)*

To begin to further explore the potential utility of P_{red}*, we calculated PRR from these values and plotted these estimates, as a whole, next to PRRs from clearance data for the artemisinins (Figure 1.7). PRRs calculated from P_{red}* were satisfactorily similar to those calculated from clearance data for artemisinin, but they were far too low compared to the average for artemether and artesunate. However, PRR for both artesunate and artemether seem to be clustered into 2 groups– one with very high PRRs, and one with

lower PRRs that seem to match the majority of those estimated from P_{red}*. A preliminary look at the covariates associated with the studies from which we obtained actual PRRs revealed that 4 out of 5 of the studies with high PRRs featured some level of complicated malaria and 2 out of 5 had some comatose patients; 2 out of the 4 lower-PRR studies had severe malaria patients, but none were in comas. Complicated malaria is usually treated differently than uncomplicated; notably, patients tend to be treated with IM injections because they cannot take medication orally. The difference in patient state, drug formulation, and drug delivery could have significant effects on pharmacokinetics and pharmacodynamics, but these distinctions fail to totally explain the wide gap in PRR values; the same trend does not hold for the similar gap in artesunate PRR values. Nonetheless, they could be suggestive of an impact from covariates. If this is the case, perhaps the estimated PRR values are, in fact, more representative of the body of data within a given drug than was initially thought.

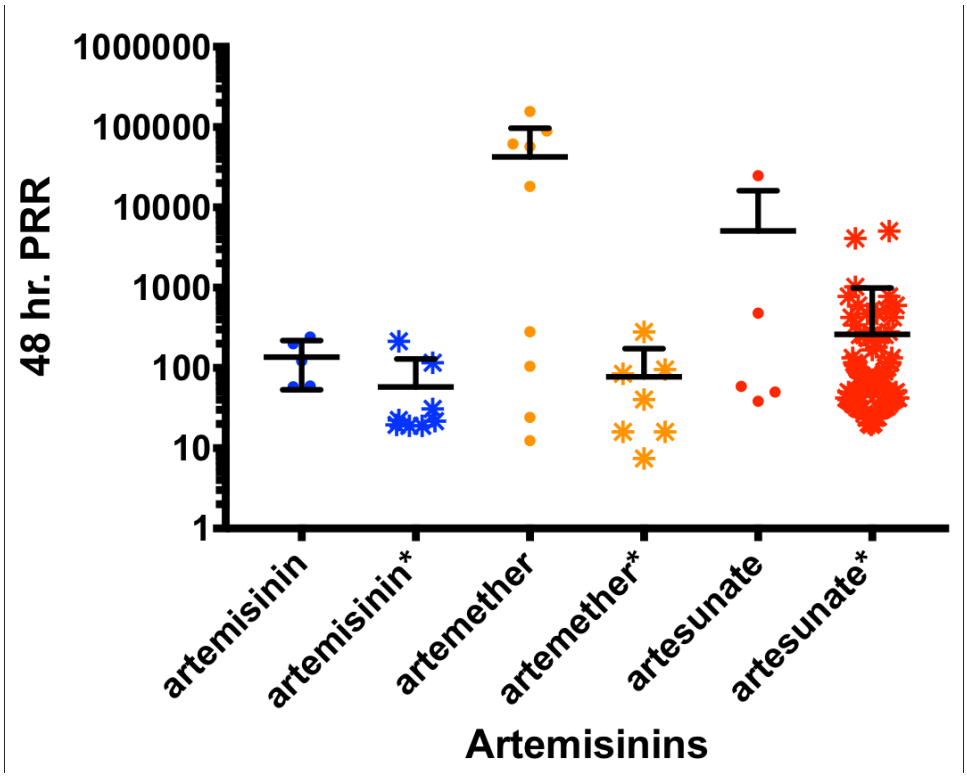

Figure 1.7 *PRR for ART, AM, and AS compared to PRR estimated from Pred* (via PC50), represented as stars.*

Lag phase

Lag time was a confounder in some drug classes, especially the antibiotics; the aminoquinolines also tend to have lag times, though not as long as antibiotics. Lag phase was present to some degree in every drug class (Figure 1.8).

The drug may be only moderately effective or totally ineffective during the lag phase, so ignoring the lag phase altogether means that the action (or inaction) of the drug before it becomes fully effective would be discounted. Instead, it was useful to look at when a drug reaches a maximum kill rate, which would factor in a lag phase should one be present. Most drugs' maximum rate (or minimum $PC_{1/2}$) centered around day 1, with artesunate and fosmidomycin acting faster and clindamycin and azithromycin much slower (Figure 1.9). The distribution of T_{max}, or the time at which clearance is the fastest,

for each drug was fitted to a Gaussian distribution by GraphPad Prism, with amplitude constrained to 1 and mean constrained to ≥0.

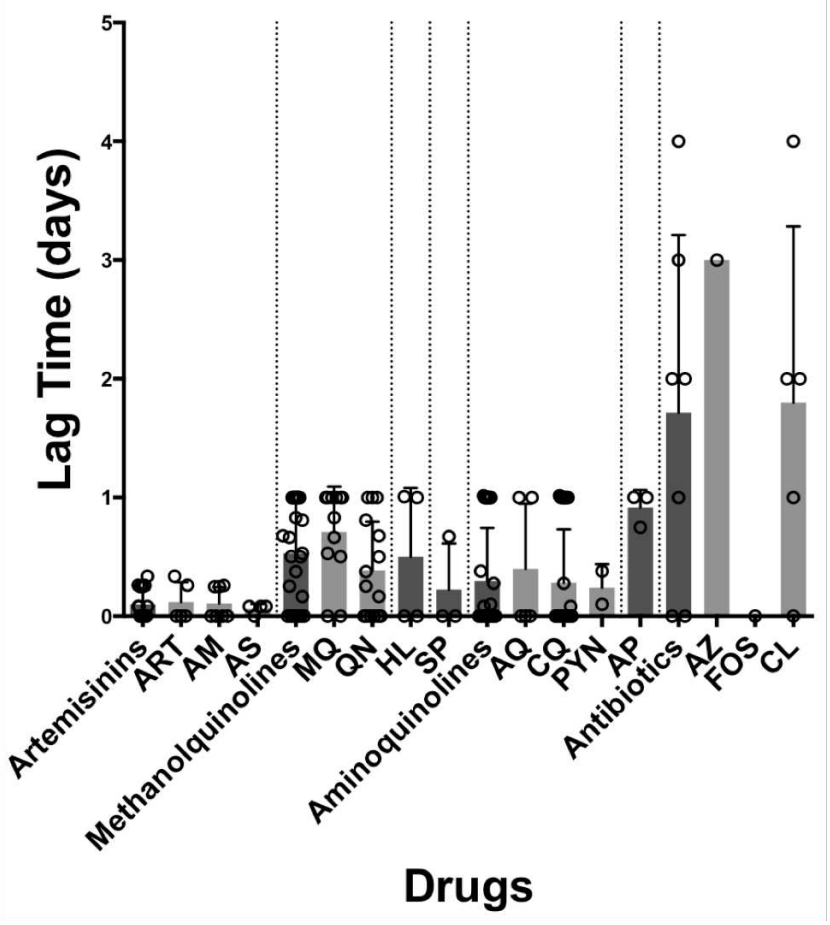

Figure 1.8 *Lag times associated with each drug and drug group.*

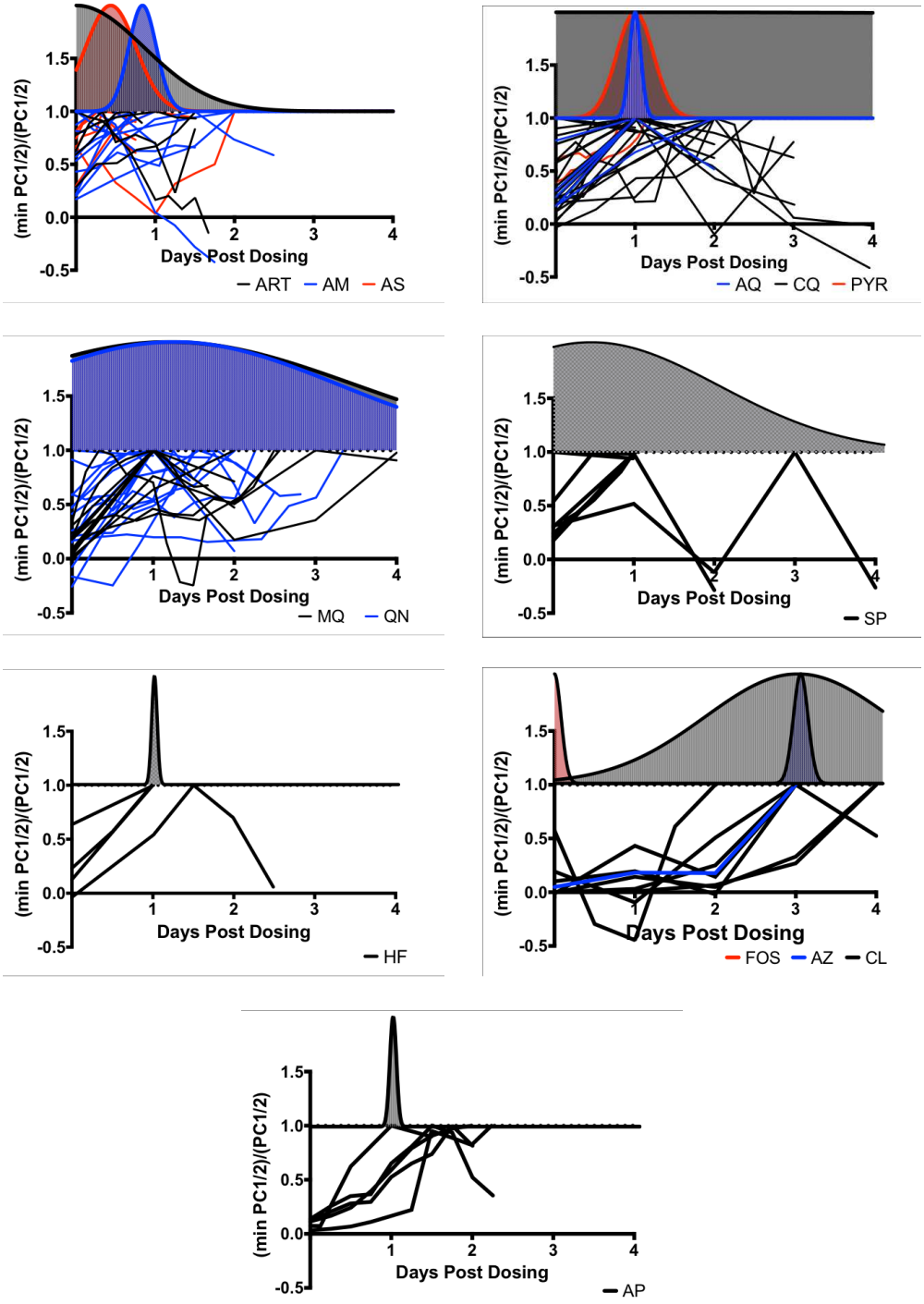

Figure 1.9 *Calculation of $PC_{1/2}$, iteratively across 24-hour spans, compared to the minimum $PC_{1/2}$ across any 24-hour span; where each line touches 1 represents the time at which clearance was the fastest. Gaussian curve above each graph indicates normalized distribution of T_{max}, or the time at which each trial is fastest, for each drug. a) artemisinins, b) methanolquinolines, c) halofantrine, d) antifolates, e) aminoquinolines, f) atovaquone-proguanil, g) antibiotics. In g), the only trial available for FOS exhibited cure within 24 hours, so only one point could be calculated.*

With the exception of the antibiotics, all drug groups tend to reach their fastest point around day 1 following treatment. Unfortunately, this is likely, at least in part, an artifact of this time point being universal across all trials. However, also interesting and potentially useful is the spread in T_{max} within a drug; for example, while the maximum kill rate of CQ occurs almost anywhere across this 4-day span, the maximum kill rate for AQ occurs fairly predictably at 1 day following treatment. Further investigation may elucidate whether the magnitude and variability of T_{max} is due to pharmacokinetics or pharmacodynamics, and this information could be useful in characterizing drugs and devising new combinations.

Geographical and temporal comparisons

PRR was stratified by region to observe any trends between Africa and southeast Asia (Figure 1.10). MQ, QN, and AM were the only drugs to be used at an appreciable level on both continents. Owing to the large standard deviation due to the use of weighted means, statistical tests failed to identify a significant difference in drug activity between either continent. However, it appears that PRR is slightly higher in Africa than southeast Asia. It is likely this difference is largely due to more ubiquitous immunity in Africa, but this may be a trend worthy of future investigation.

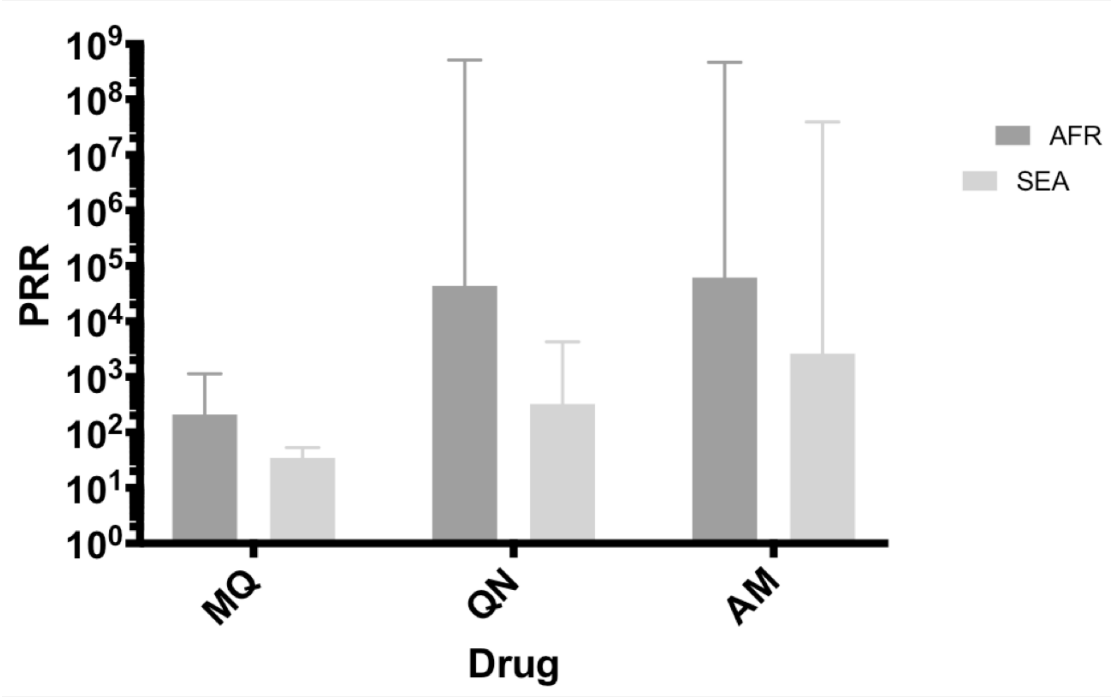

Figure 1.10 *PRRs of MQ, QN, and AM in both Africa (AFR) and southeast Asia (SEA).*

Most drugs exhibited an increase in PRR throughout time, which runs counter to that which may be expected with the development of resistance to some drugs (Figure 1.11). Hastings *et al* (2015) argued that clearance rates are insensitive probes for resistance, which may explain this negative finding; alternatively, these results could be subject to bias due to the fact that a drug is less likely to be given as monotherapy once resistance is suspected.

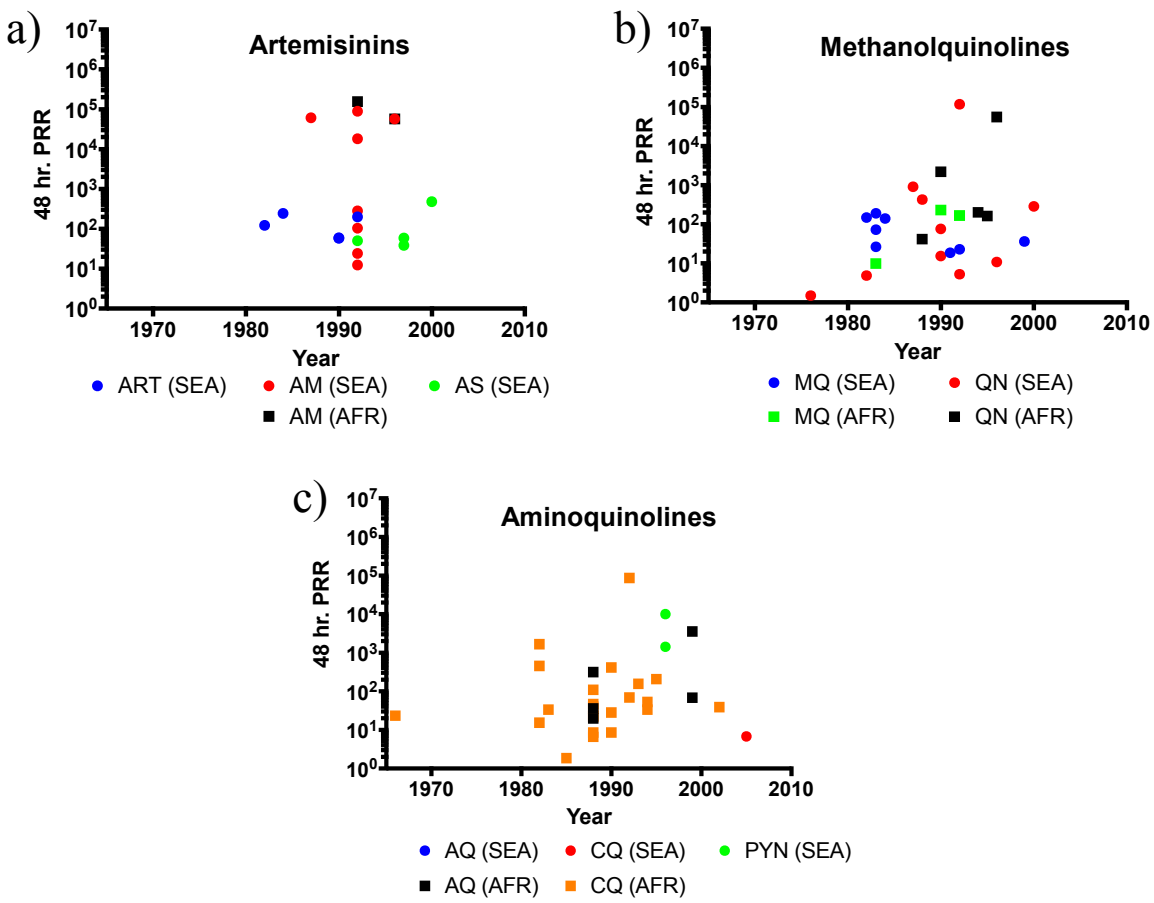

Figure 1.11 *PRR through time in both Africa (AFR) and southeast Asia (SEA); only MQ in southeast Asia, ART in southeast Asia, and AM in Africa exhibited decreases in PRR through time; a) artemisinins, b) methanolquinolines, c) aminoquinolines.*

Discussion

The discussion of the data, like the the introduction, will be broken into three sections, each dedicated to different, yet equally important observations made throughout the collection and analysis of this dataset.

The anatomy of the malaria monotherapy trial

The usability of the results in the average clinical trial report left much to be desired. Many publications reported no measure of parasite clearance whatsoever, and for those that did, there was no universal metric. In addition, there was no universal

measures of central tendency or error either, making comparison across trials difficult. While logistical concerns with the data collection may, to some extent, dictate the types of results that can be reported, the measures of central tendency and error should be ubiquitous. We recommend geometric mean and standard deviation for all measures of parasite density, and mean and standard deviation for all other measures.

Pharmacodynamic results

Most of our results are relatively unsurprising given the empirical rules of thumb already known to those that utilize and research malaria therapeutics. The trends we found in the magnitude of PRR and PCT were largely what we expected; the variation within each drug, however, warrants further examination. In addition, we tried to devise more robust metrics to utilize more of the body of clinical trial data, but these new measures need fine-tuning. Other analyses, such as the consideration of the timing and magnitude of the maximum kill rate for each drug, were relatively unique and could provide quantitative insight into the pharmacodynamic actions of a drug.

Parasite Reduction Ratio

While our results are the product of analyzing an amalgamation of trials featuring different populations, routes of administration, and other covariates, the average PRR values for each drug are relatively unsurprising compared to that which is traditionally expected. For example, artemisinin and its derivatives have been touted as capable of a 10^4-fold drop in parasite count over a single life cycle. While artesunate and artemether occasionally reach this threshold, artemisinin does not. Perhaps surprisingly, quinine and chloroquine also sometimes reach this level.

Parasite reduction ratio remains the primary metric for describing parasite clearance rates, though its merits have been questioned. For example, PRR is statistically unrelated

to the growth of the parasite during the first few hours immediately following drug administration, during which time parasite growth is known to be more closely associated with growth patterns prior to treatment than the identity of the drug administered (Hastings *et al.,* 2015). Nevertheless, at this time, PRR must be sufficient for at least qualifying, if not quantifying, drug kill rates. Based on this measure, it is clear that the artemisinin derivatives are associated with the highest PRR despite the wide variation.

Parasite Clearance Times

Time to absent detectable parasitemia (PCT), while potentially useful within trials for comparing study arms, is subject to many variables that make it less reliable as a comparator between trials. Determination of the time at which parasites have been cleared will always be subject to some random action at the tail end of the parasite clearance curve, as the dwindling parasite population and/or drug concentration affect the predictability of their successful interaction. Additionally, the resources available to the researchers may vary across studies and could exacerbate any uncertainties. The use of thick or thin blood smears, the experience of the microscopist, and the number of fields observed are some factors that may not be universal across many studies. The use of PCR makes observation much more sensitive, and thus, will extend PCT.

We feel that PC50 is a somewhat more reliable metric for quantifying clearance times. While it it less clinically relevant, the larger number of parasites means that we should feel more confident in the relative accuracy of the measurements. Also, for better or worse, it is less likely to be related to antimalarial resistance. For our analyses, we prefer measurements that are less affected by resistance, as we were interested in considering the potential of a drug, not its actual effectiveness, which will vary according to geographic and temporal variables that have not been considered yet.

New Metrics

Unfortunately, none of the metrics devised to describe the parasite clearance curve are sufficient proxies for clinically-observed data. While these measures could be used for modeling the parasite clearance curve, as of now they are far too rudimentary and would need to be adjusted at least for each drug or drug class; factors such as delivery method, host immunity, and dose would almost certainly need to be included as well.

For now, $PC_{1/2}$ is best suited as an empirical descriptor of the parasite clearance curve, like PRR. However, while $PC_{1/2}$ has more opportunity to be adjusted to provide a mechanistic model of the clearance curve, PRR is more firmly entrenched in the literature, and, at the current juncture, $PC_{1/2}$ does not provide any additional utility. P_{red}* is based on the same assumption as $PC_{1/2}$, and therefore should not be used in an explanatory capacity until it can be adjusted for covariates.

Lag Phase

The lag phase is an interesting characteristic of the parasite clearance curve that has been described in greater theoretical detail elsewhere (Khoury *et al.,* 2016). We found that every drug class underwent a lag phase in some trials, which runs counter to the traditional classification of "fast" and "slow" acting drugs, but nonetheless conforms with the observation that even parasites treated with fast-actors are influenced to a great degree by pre-treatment growth patterns.

While artemisinins are considered the preeminent "fast-acting" antimalarials, we observed discrepancies in the precise time that artemisinin and its derivatives actually reach their peak effectiveness. While it has been noted previously that measurements of clearance are subject to bias based on splenic clearance of dead and dying parasites

(Stepniewska *et al.,* 2010), it can be assumed that all of the studies in these analyses are subject to similar biases and are therefore comparable, if not exactly numerically accurate.

It is our hope that such characterizations of lag may be useful in the future when trying to determine ideal antimalarial combination therapies. The time of action of each drug alone, assuming it is related to the same measure when in combination with another drug, would be central to any combination therapy.

Initial parasitemia

Our results show no clear correlation within any drug group between PRR and initial parasitemia, but this relationship will be explored further. Stratification drastically reduced the sample size, particularly for some drugs where data were already sparse. In addition, parasite counts may decrease slightly even in severe cases of malaria due to quorum-sensing, immune activation, or sequestration. As such, while we can draw no conclusions regarding correlations between initial parasitemia and kill rate, we may not have been able to capture the progression of the infection by using initial parasitemia alone.

The utility of the results

We acknowledge that the explanatory power of these analyses is severely limited by the wide variation in a vast array of covariates. Nevertheless, we feel that our results throw into question the canonical kill rates associated with each drug or drug class. While we would not criticize the ranking of each class according to average clearance rate, the value of the rate may both oversimplify the variation inherent in responses to treatment and inflate the rate to that of the best case scenario– most all trials featured rates approaching, but never quite reaching, these expected values.

One potential application of this study would be an attempt to identify new combination therapies– perhaps including drugs analyzed here, or perhaps including a drug analyzed in the same method as the current study. Hastings and Hodel (2014) described in some detail the various parameters that need to be considered when designing a new combination. While many refer to the interaction between the constituent drugs, which would not be described adequately by these analyses, the fact remains that the better we can describe the action of a drug, the better we can deploy it.

An attempt to fit these results and data into the existing literature may be a mischaracterization of the goals of this endeavor. Instead, we set out to collect the data around which other hypotheses in the field of malaria chemotherapy would be based. We acknowledge that this aim is far from complete, with many studies yet to be included, drugs yet to be characterized, and variables yet to be analyzed. Nonetheless, the results currently presented represent an affirmation of some paradigms widely embraced in malaria chemotherapy– namely, which drugs can be considered the fastest actors– as well as a reality check on others– for example, how some drugs rarely live up to their billing with respect to PRR, but nearly all drugs have a wide variability in kill rates.

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Chapter 2: The effect of the stage of growth of *P. berghei* parasites on antimalarial kill rates

Introduction

Numerous studies have identified antimicrobial resistance or decreased antimicrobial kill rate in bacteria in biofilms (Maira-Litrán *et al.,* 2000; Brooun *et al.,* 2000; Das *et al.,* 1998) or in the stationary phase of growth (Eng *et al.,* 1991). It has thus been hypothesized that physiological changes associated with quorum-sensing, including altered gene expression and the induction of a stress response, mediate this reduced sensitivity to antibiotics (Mah and O'Toole, 2001; Brackman *et al.,* 2011; Hentzer and Givskov, 2003; Udekwu *et al.,* 2008). The interaction between *Plasmodium* parasite density and antimalarial kill rates has yet to be observed.

In Chapter 1, we showed that there has been no clear relationship between initial parasitemia and kill rate within or across drugs or drug groups in *P. falciparum* monotherapy clinical trials. However, the stratification performed in that analysis may be insufficient in that parasitemia is not strictly correlated with clinical status. This could confound our previous analysis in two distinct ways, both described in the review article by White (1997). First, relatively naïve patients could present with severe clinical malaria yet have a parasitemia undetectable by thin blood film; meanwhile, highly immune patients can exhibit asymptomatic hyperparasitemia. If the pharmacokinetics or pharmacodynamics of a drug are affected by the patients clinical status, this would not be captured entirely by stratification of parasitemia because of the wide array of populations included in the analysis. Second, following a phase of exponential growth, parasite counts in patients with untreated *P. falciparum* infections subside somewhat and reach a plateau. This subsidence means that patients with parasites in the exponential and plateau

phases may be stratified together, despite the probability that the differing physiology of these parasites alters pharmacodynamics.

In this light, the question remains whether parasite density has any bearing on kill rate following treatment with antimalarials. Parasite clearance immediately following treatment tends to follow a first-order decay pattern, suggesting that parasite density itself should not drive any discrepancy between early- and late-treated parasites. The reduction in effectiveness of antibiotics on bacteria at high densities is attributed to quorum-sensing. A two-component system for response to environmental changes has been observed in intra-erythrocytic *P. falciparum* parasites (Wu *et al.,* 2016), so similar physiologic changes could beget delayed parasite clearance following treatment during the plateau phase. Other explanations for this phenomenon could include those hinging on the clinical status of the subject, like splenic or immune function; the stress response of the parasite (Dogovski *et al.,* 2015); the shifting preference for host cell from normocytes to reticulocytes during late-stage infection (Singer, 1954); or the interaction between the parasite and host in a changing oxidative milieu (Becker *et al.,* 2004).

In order to identify a relationship between the progression of the *Plasmodium* infection and the initial kill rate following treatment, we used a murine model infected with *P. berghei* and treated at one of several points throughout the progression of the parasitemia. In doing so, we are able to observe differential parasite clearance curves, as well as compare outcomes, according to the progression of infection while controlling the covariates that likely compromised the previous analysis of human data.

Methods

Study design

All mice used for this experiment were naïve BALB/C mice from Jackson Labs between 6 and 8 weeks old. Mice were housed in cages with no more than 5 mice per cage, kept at approximately 76ºF, and provided food and water *ad libitum*.

All mice were infected with *Plasmodium berghei* ANKA strain parasites expressing the GFP-luciferase reporter and were treated with a single drug in a cytocidal model. Parasites were low-passage (*i.e.*, less than 5 passages through mice before being passed through mosquitoes) in order to ameliorate the effects of selection on the parasites. Donor mice were either infected directly from a frozen stock or were untreated mice from previous experimental arms. In the latter case, 500,000 parasites were transferred from the donor to each naïve mouse. In most cases, the parasites transferred from the donor would have been in the plateau phase, but were given ample time before treatment to establish log growth in the recipient mice. The passage number of the parasites through mice for each experiment arm is depicted in Figure 2.1. All study arms contained groups of three mice. Inoculation of naïve mice from donors that had been infected for at least 6 days, as well as the observation from Vanderberg (1982) justify our assumption that all infections were asynchronous.

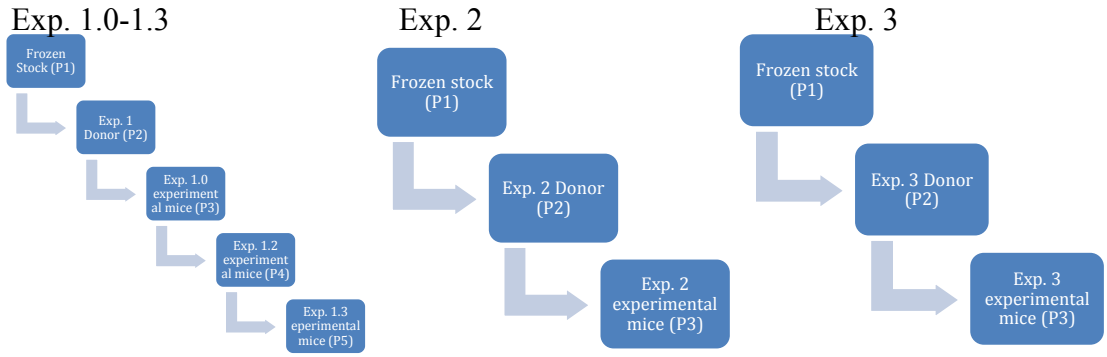


Figure 2.12 *Passage (P) of parasites through mice for each experiment*

The actual allocation schedule and parasite counts for all mice at the time of treatment are listed in Table 2.1.

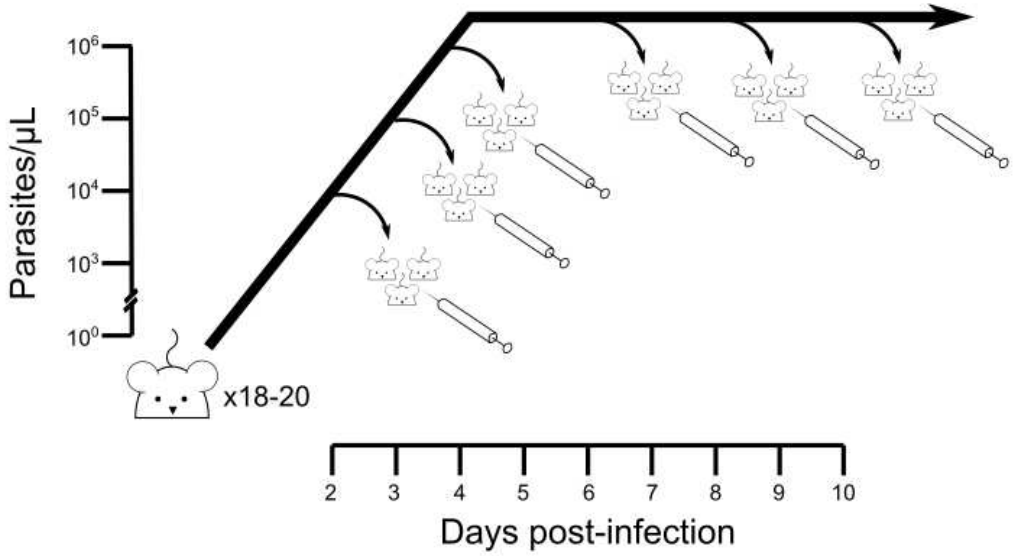


Figure 2.13 *Projected allocation and dosing schedule, arrows represent allocation and initiation of treatment during Experiment 1; in Experiments 2 and 3, mice were only allocated to treatment groups on day 4 or day 8 after treatment. Following the inflection point between log phase and plateau phase (often about day 4-6 following infection), there is more variation in the pattern of growth. In some study arms, the projection needed to be translated horizontally to accommodate slower-progressing infections, but the pattern remained applicable.*

		Starting parasite counts at time of initiation of treatment (parasites/μL)					
Exp. number	**Treatment**	**~2 days post-infection**	**~3 days post-infection**	**~4 days post-infection**	**~6 days post-infection**	**~8 days post-infection**	**~10 days post-infection**
1.0	AS	*Day 3*	*Day 3*	*Day 4*	*Day 6*	*Day 8*	*Day 10*
		14584.7	120792.4	1000597.5	215098.5	1149490.7	3293605.2
1.1	AQ		*Day 5*	*Day 5*	*Day 7*	*Day 9*	*Day 11*
			295456.9	1310800.5	196791.2	228942.2	6197825.8
1.2	PYN	*Day 3*	*Day 4*	*Day 5*	*Day 7*	*Day 9*	*Day 11*
		10292.4	92271.7	152340.4	862990.7	6910700.4	5029720.9
2	AS			*Day 4*		*Day 8*	
				111772.0		362736.1	
	AQ			*Day 4*		*Day 8*	
				77732.8		405735.3	
	PYN			*Day 4*		*Day 8*	
				115715.7		466588.6	
3	AS			*Day 7*		*Day 11*	
				1725070.2		15783932.6	
	AQ			*Day 7*		*Day 11*	
				1503959.7		10718013.4	
	PYN			*Day 7*		*Day 11*	
				1553873.9		11910179.4	

Table 2.5 *Parasite counts (in parasites/μL, measured by luciferase assay) at the time of treatment initiation for all treatment groups in all study arms. The parameters by which the groups were allocated are estimates based on the expected growth of untreated parasites. The exact timing of dosing is described in the methods for each experiment. Within each band, the cells in the top row are the day on which the mice in that group actually initiated treatment, while the bottom cells are the geometric mean of the 3 mice in each group.*

The mice in these studies were treated with monotherapy regimens of either artesunate, amodiaquine, or pyronaridine. The drugs were prepared from powder stocks. Artesunate was dissolved in 5% sodium bicarbonate, while amodiaquine and pyronaridine were dissolved in deionized water. The solutions were refrigerated until use. All doses were administered via intraperitoneal injection.

Data collection and analysis

All mice were sampled once daily for 14 days following treatment and then every other day until at least day 30 following treatment. On the first day of drug dosing, unless otherwise noted, mice were sampled concurrently with treatment and then at 3, 6,

and 12 hours following treatment. Additional samples or slight deviations from this schedule were noted and are reflected in the data presented.

We collected 5 µL of tail blood at each sampling point and immediately added it, in a 1:9 ratio, to lysis buffer (20 mM Tris, 5 mM EDTA, 0.008% saponin, 0.08% Triton x-100; pH=7.5) in a 96-well plate for analysis by luciferase assay. For analysis of the samples, 5 µL of this solution was added to 95 µL of a luciferase buffer (200 mM Tricine, pH=7.8; 10 mM EDTA, pH=8; 10 mM K_2CO_3, pH=7.8; 50 mM $MgSO_4$; 250 mM DTT; 25 mM ATP; 20 mM D-kuciferon) and photon emissions were recorded by the IVIS Spectrum In Vivo Imaging System and analyzed using Living Image software (v. 4.4). Until the assay was run, plates were stored at -76ºC. A blood film was created once daily for each mouse (on days where blood samples were taken), regardless of its treatment status. These blood films were stained with Giemsa stain and were used to corroborate luciferase assay results. Parasite counts were extracted from the results of the luciferase assay according to the standard curve described elsewhere (Walker and Sullivan, 2017).

The results were analyzed in GraphPad Prism by observation of raw clearance curves, as well as normalization to 100% of initial parasitemia. In addition, Kaplan-Meier survival curves were produced and t-tests calculated using GraphPad Prism (GraphPad Prism version 7.0b for Mac).

Experiment 1

The first experiment, featuring three separate drug arms, with mice treated at 10,000, 100,000 and 1,000,000 parasites/µL at roughly days 2, 3, and 4 post-infection for the log phase (Figure 2.2) as well as “late-treated” mice dosed during the plateau phase of

parasite growth on approximately day 6, 8 and 10 with parasitemia in the million parasites/μL range.

Experiment 2 and 3

The protocol for the second experiment was designed based on the results from the first; rather than treating throughout the progression of the infection, we decided to simply treat mice on day 4 (between 50,000 and 500,000 parasites/μL) or day 8 (greater than a million parasites/μL) post-infection before or after the inflection point between the log growth and plateau phases with different drug doses to see if the effect was enhanced.

Results

The parasite counts at the time of treatment initiation, shown in Table 2.1, were comparable across and within all drug treatment groups for which treatment began at the same time. The parasite growth in untreated mice is similar to that in humans, as described by White (1997); rapid expansion of the parasite population occurs over the first several life cycles at a roughly log-linear rate, and then growth slows and plateaus as parasite counts reach a certain threshold. In mice, this threshold is about 5-10 million parasites/μL. During this time, parasite counts may rise or fall slightly, but there is little change overall. Log growth resumes around day 8-10. Log and plateau phases are clear in all three groups in Experiment 1, with the transition from the former to the latter occurring at some point between the 4^{th} and 6^{th} day following infection (Figure 2.3). Untreated mice in Experiments 2 and 3 followed the same pattern (not shown).

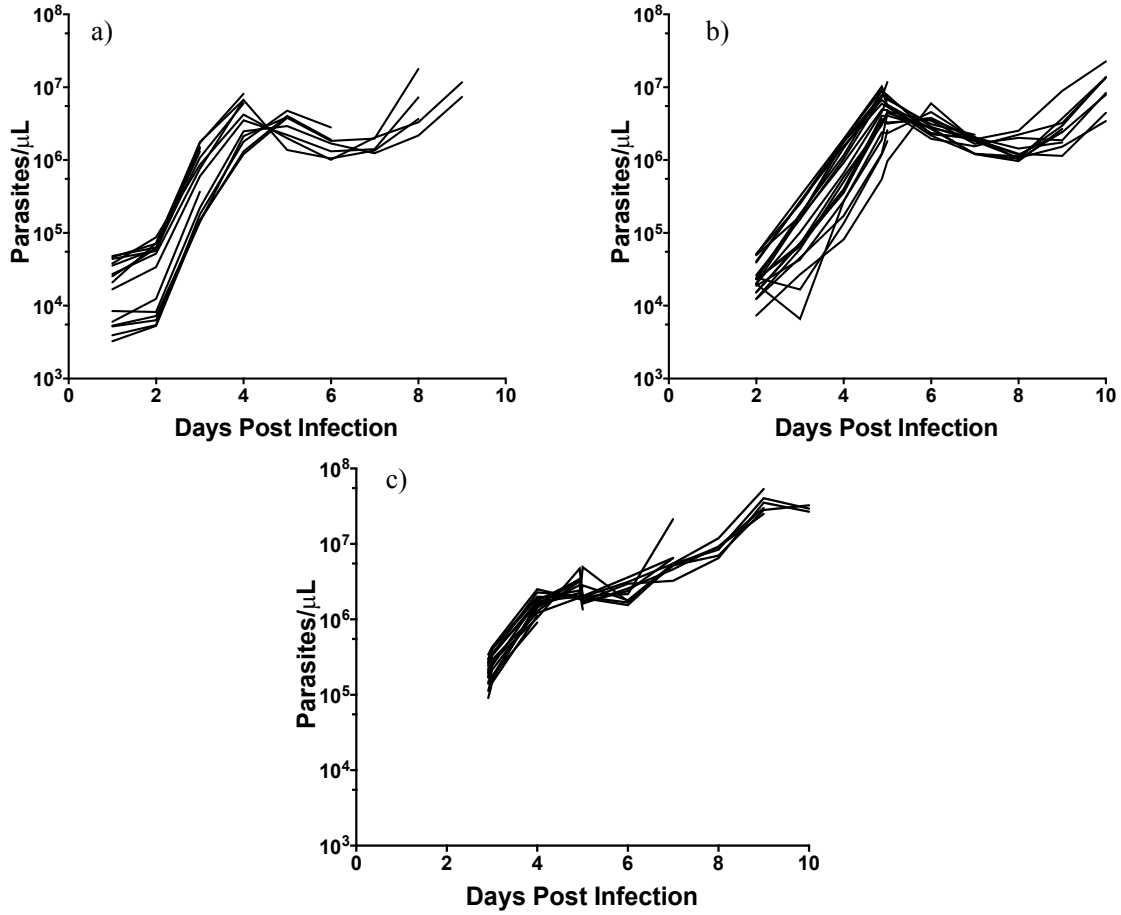


Figure 2.14 *Parasite growth of untreated mice in a) Experiment 1.0, b) Experiment 1.1, and c) Experiment 1.2. The curve for each mouse is truncated at the time of initiation of treatment.*

In most treatment groups, mice treated during the plateau phase exhibited slower clearance rates initially (Figure 2.4-7). The discrepancy between the clearance curves of early- and late-treated mice tends to be visually apparent, and often reaches statistical significance at some early time points (α=0.05). The difference in kill rates, however, does not translate to a difference in outcome.

Geometric means and geometric standard deviations among all three mice in a group (or those surviving to that point) are shown at each time point in every graph, while statistical tests were only performed on graphs featuring two clearance curves– one for an early-treated group and one for a late-treated group.

Experiment 1– 50mg/kg AS at 0 and 24 hours; 120mg/kg AQ at 0 and 24 hours, 60mg/kg AQ at 48 hours; or 60mg/kg PYN single dose

The raw data, in parasites/μL, show a clear distinction in clearance times between early- and late-treated groups in mice treated with PYN, but the time to reach the limit of quantification is more uniform in the AS and AQ groups despite early differences in the slope of the clearance curve. Despite apparent differences in clearance rate or clearance time, the overall number of parasites cleared does not appear to be affected, except in the case of the hyperparasitemic mice treated with AS (Group 6). In order to better compare clearance rates, which are not correlated with initial parasitemia, the remainder of the results in this report will present a parasite count normalized to 100% of initial parasitemia for each group.

Following normalization, the discrepancies in clearance rates between early- and late-treated mice becomes apparent in those treated with AS. While the difference in slope is smaller in the AQ and PYN groups, the early-treated groups do have a slightly faster clearance rate than the late-treated groups over the first several hours (Figure 2.4). Group 4 in the AQ-treated mice, which were treated on day 7 following infection, are the obvious exception to this general trend.

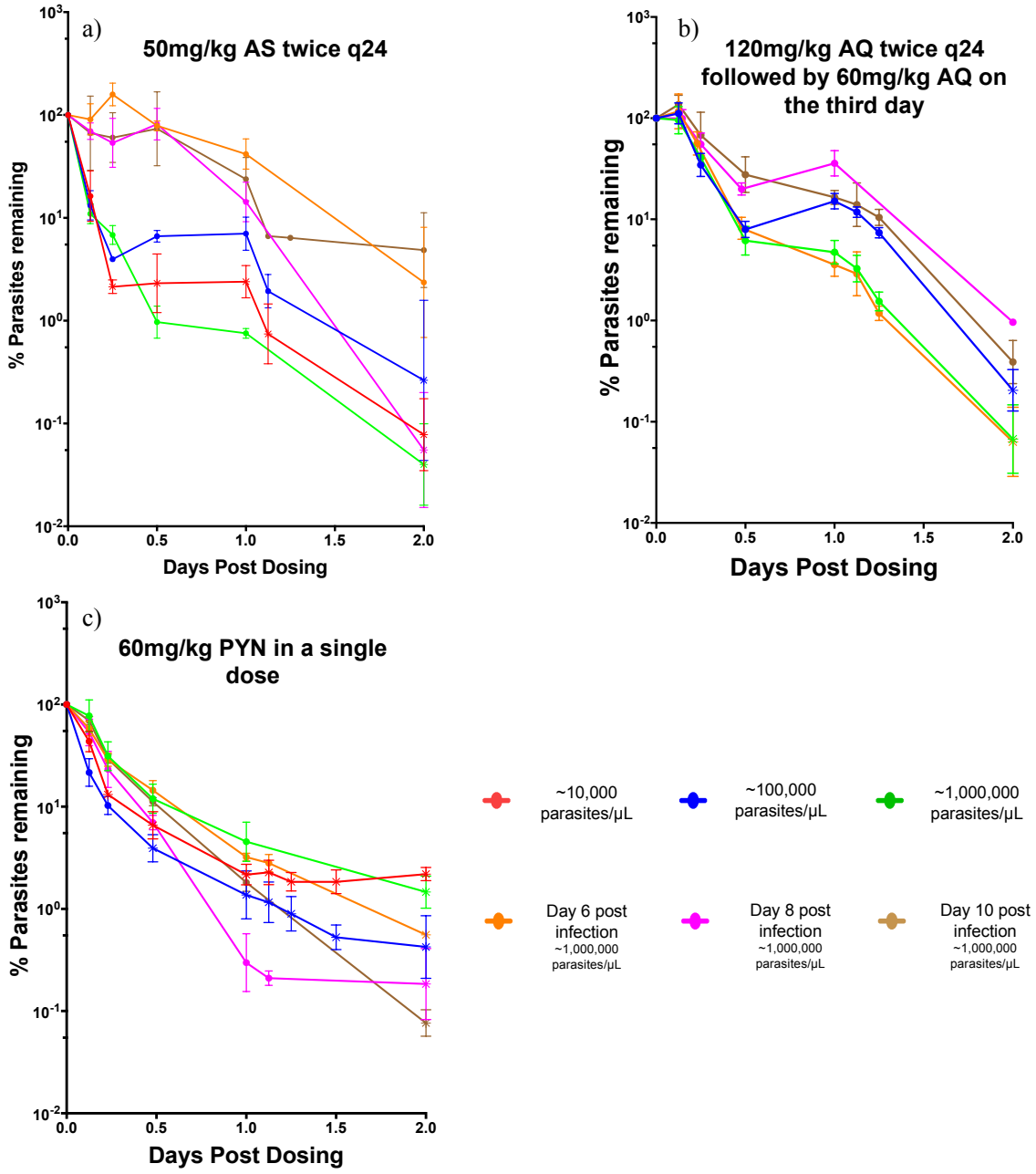


Figure 2.15 *Parasite counts, normalized to 100% of initial parasitemia, for mice treated at various points along the progression of infection for the first two days following treatment with a) 50mg/kg AS twice q24, b) 120mg/kg AQ twice q24 followed by 60mg/kg AQ on the third day, or c) 60mg/kg PYN in a single dose. The parameters listed in the figure legend by which the groups were allocated are estimates based on the expected growth of untreated parasites. The exact timing of dosing is described in the methods for each experiment; groups should be considered comparable across drugs.*

These data, while demonstrating a distinction in clearance rates between early- and late-treated mice, failed to exhibit parasitemia-dependent kill rates. We felt that it

would, therefore, be sufficient to treat mice only on day 4 (during the log-growth phase) or day 8 (roughly the same parasitemia during the plateau phase).

Experiments 2 and 3

The results of Experiment 1 warranted further investigation into the difference in clearance rates of early- and late-treated mice. However, the apparent clustering of early- and late-treated groups suggested that we need not observe clearance rates at various steps though the infection of progression (Figure 2.4), but instead only need choose representative treatment start times from the log-growth and plateau phases. The results in Figure 2.5 are clearance curves of only selected groups from Experiment 1 with the results of t-tests performed on each point.

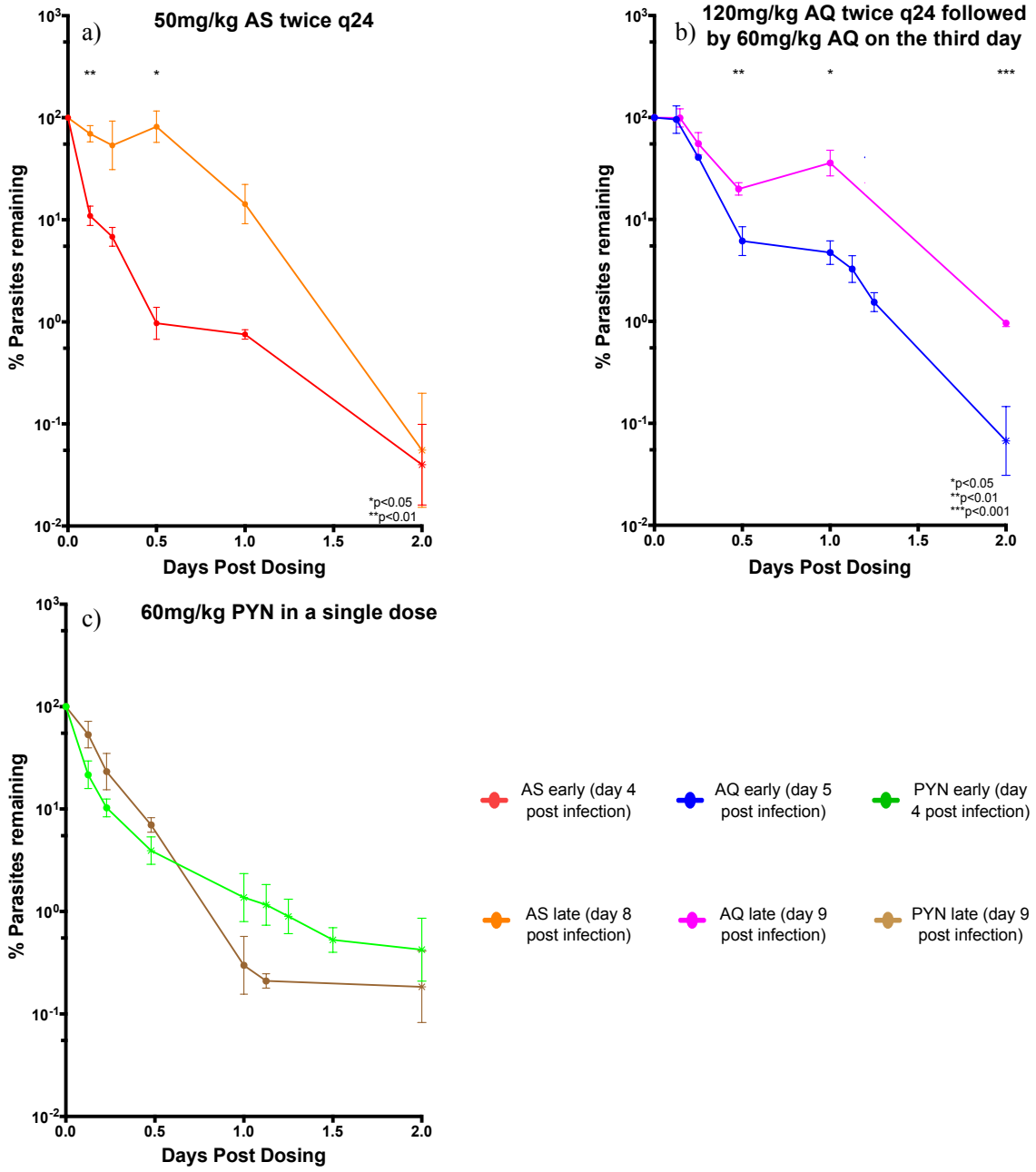


Figure 2.16 *Selected clearance curves from Figure 2.4 illustrating discrepancies in clearance rates depending on whether treatment was initiated during log-growth or plateau phase. a) 50mg/kg AS twice q24, starting on either day 4 or day 8 following infection; b) 120mg/kg AQ twice q24 followed by 60mg/kg AQ on the third day, starting on either day 5 or day 9 following infection; or c) 60mg/kg PYN in a single dose, starting on either day 4 or day 9 following infection.*

We felt that lowering the doses of the quinolines may help distinguish early- and late-treated mice. Therefore, the study arms in this experiment feature intermediate

(Experiment 2) and low (Experiment 3) doses of quinolines. Because AS treatment already exhibited a significant discrepancy between the early and late groups, and because 50mg/kg was already non-curative, we did not lower the dose of AS administered; instead, we altered the regimen such that the mice received more frequent doses (Experiment 2) or a single dose (Experiment 3).

Experiment 2– 50mg/kg AS at 0, 8, and 16 hours; 50mg/kg AQ single dose; or 10mg/kg PYN single dose

At the intermediate quinoline doses used in this study arm, there appeared to be a far less marked distinction between early- and late-treated groups; similarly, more frequent AS dosing may have mitigated the effect we observed previously (Figure 2.6). Nevertheless, the difference in percentage of parasites remaining was large enough to reach statistical significance at one or more time points in the first 4 hours in each treatment group. Once again, the timing of the initiation of treatment was unrelated to the recurrence of parasitemia or survival outcome.

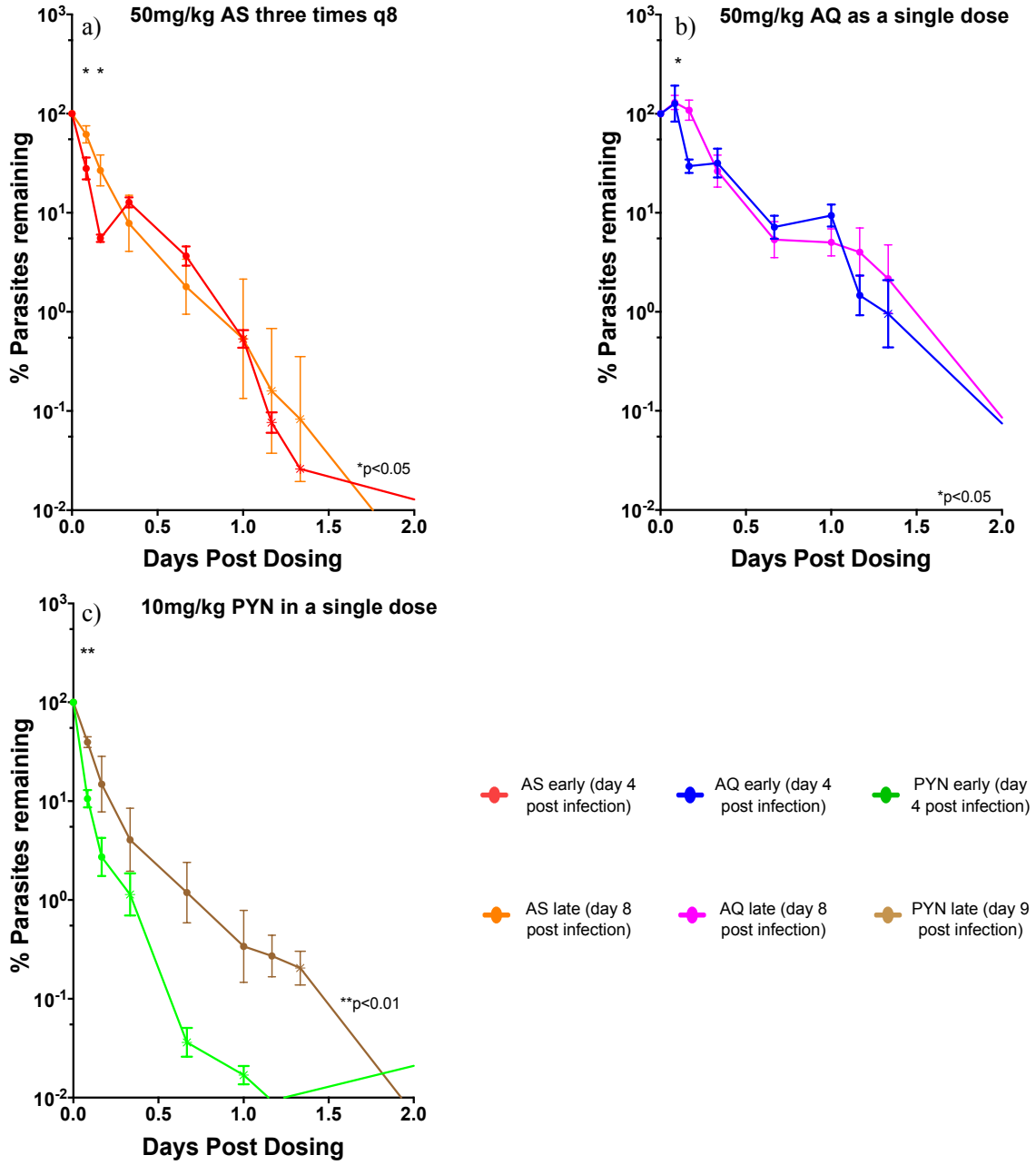


Figure 2.17 *Percentage of parasites remaining following treatment during the log-growth phase (4 days after infection) or plateau phase (8 days after infection). Mice were treated with a) 50mg/kg AS three times q8; b) 50mg/kg AQ as a single dose; or c) 10mg/kg PYN as a single dose.*

Experiment 3– 50mg/kg AS single dose; 10mg/kg AQ single dose; or 5mg/kg PYN single dose

A further decrease in dose again showed a difference in initial kill rate without an associated change in recrudescence or outcome (Figure 2.7). Because these doses are

sub-curative, there is somewhat more variation in the magnitude of the response at later time points, but statistically significant differences in clearance rates were obtained in AS- and AQ treated mice within the first 2 days after treatment; as with Experiment 1, though PYN-treated mice did not reach a statistically significant discrepancy between early- and late-treated mice, the early-treated group nevertheless appears to have cleared

parasites faster.

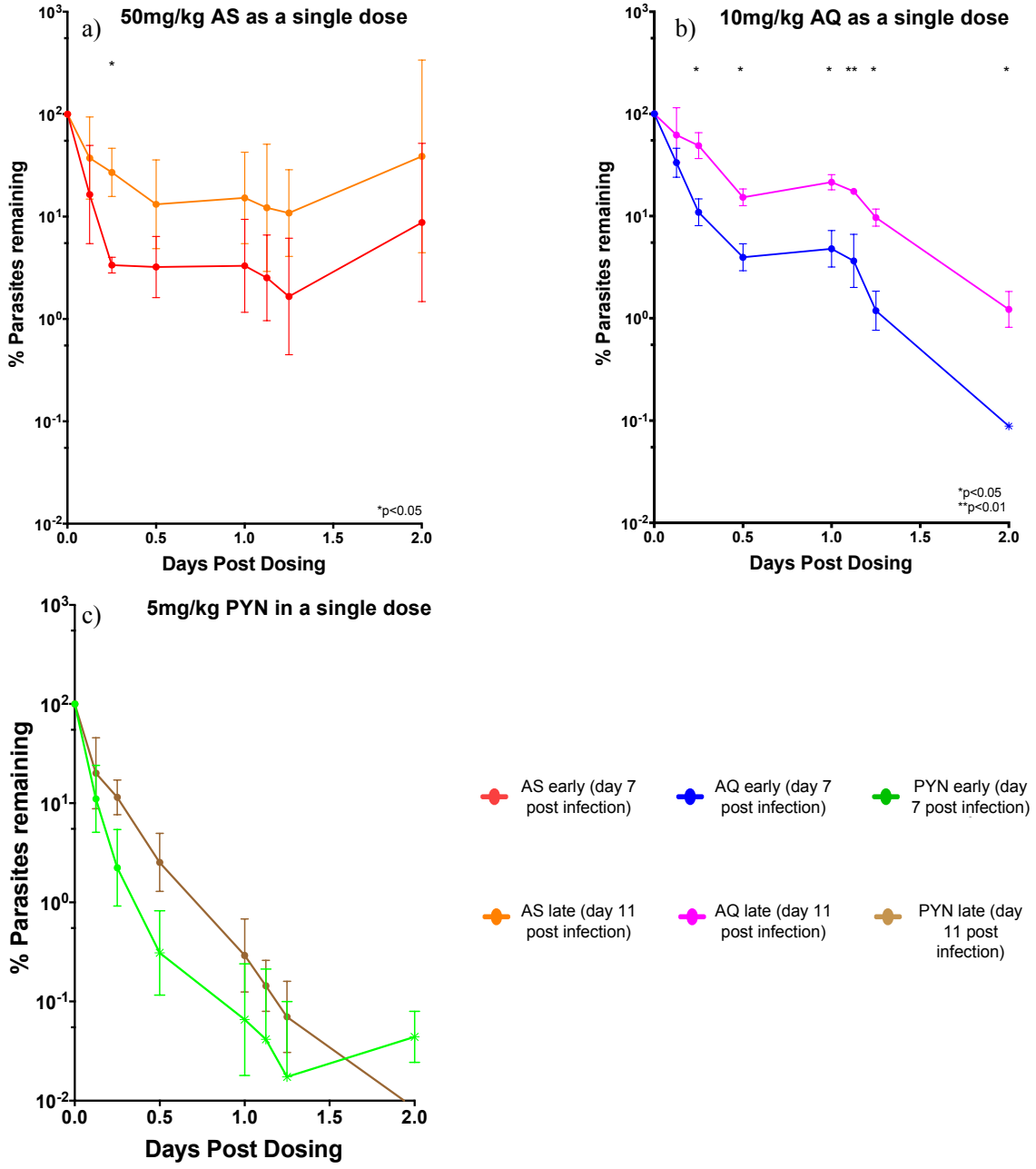


Figure 2.18 *Percentage of parasites remaining following treatment during the log-growth phase (7 days after infection) or plateau phase (11 days after infection). Mice were treated with a) 50mg/kg AS as a single dose; b) 10mg/kg AQ as a single dose; or c) 5mg/kg PYN as a single dose.*

Combined results

Because two AS-treated groups showed clear differences between early- and late-treated groups, while the AS-treated mice in Experiment 2 were somewhat more similar, we chose to pool these data to see if the aggregated results still showed the distinction. Our observations only extend for the first 8 hours after treatment, because after this point the treatment regimens began to differ. We used data from groups 3 and 5 from Experiment 1.0, as well as all of the AS data from Experiments 2 and 3. Despite the different doses, each early-treated mouse had cleared a greater percentage of its parasites than each late-treated mouse at every time point, (Figure 2.8).

Using GraphPad Prism, we performed a non-linear regression with the y-intercept constrained such that it was equal to 100 for each group. We then compared the slopes of the resulting log-linear lines using a sum-of-squares F test. The slopes of the early- and late-treated groups are statistically distinct ($p<0.0001$). In addition, the r^2 value of the late-treated group indicates a much poorer correlation of these values (Figure 2.8). The same analyses were performed for the AQ- and PYN-treated groups. While these early- and late-treated groups were not as divergent as in the AS groups, the early clearance rates in late-treated groups still seemed to be both slower and less uniform than the early-treated groups.

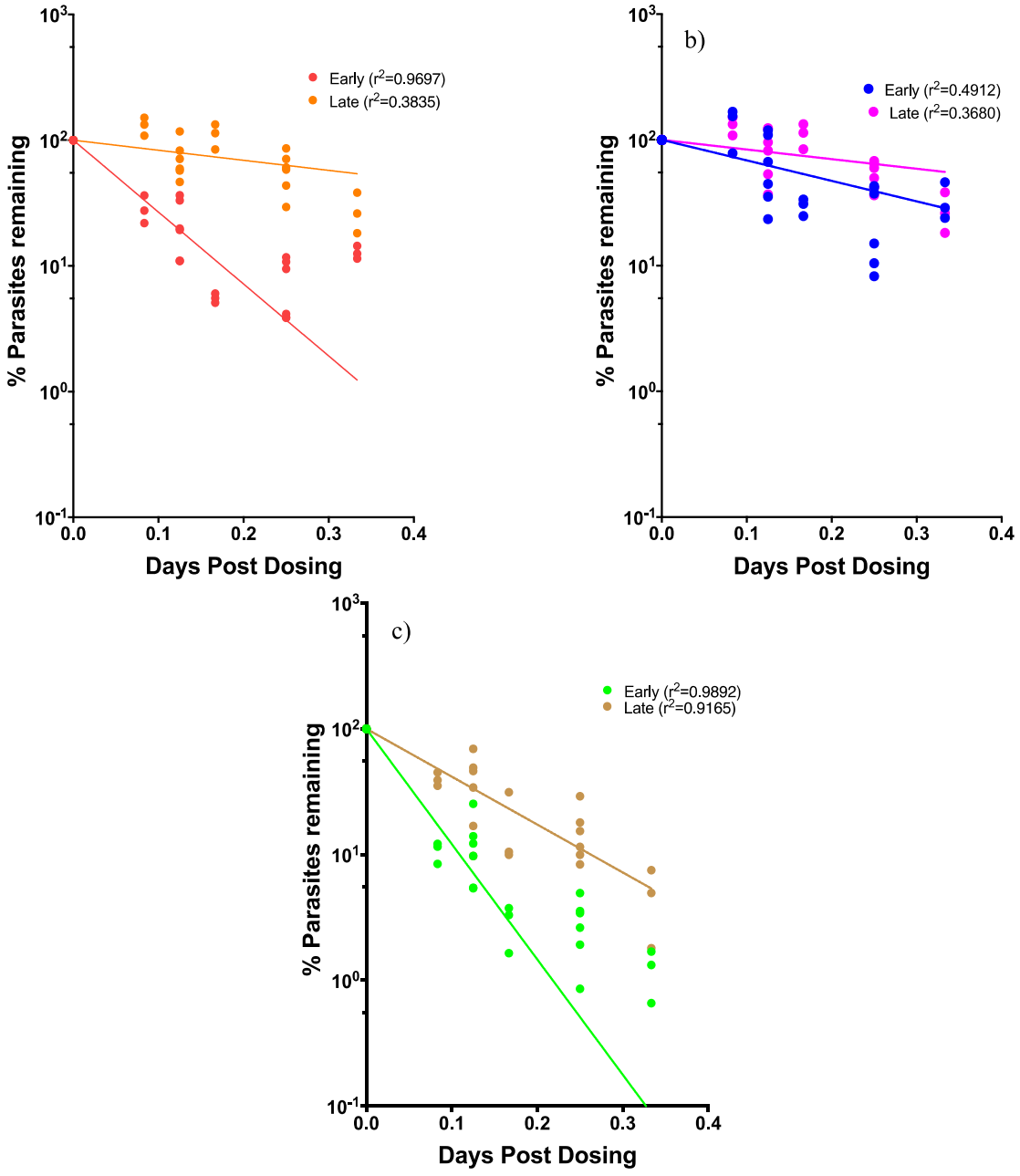
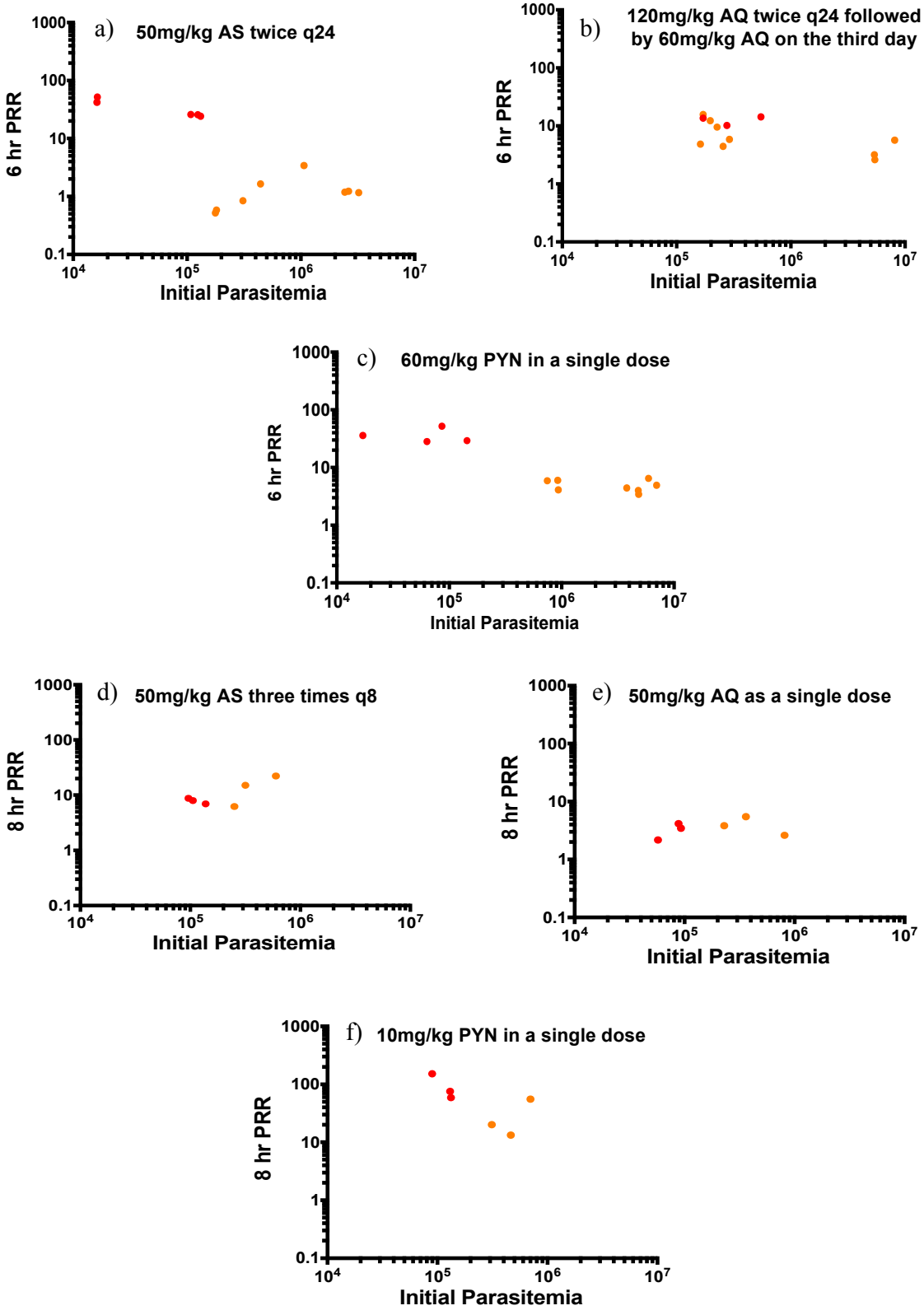


Figure 2.19 *Pooled data from the first 8 hours following treatment in a) AS-treated mice from Experiment 1.0 (early: treated on day 4; late: treated on day 8), Experiment 2 (early: day 4; late: day 4), and Experiment 3 (early: day 7; late: day 11); b) AQ-treated mice from Experiment 1.1 (early: treated on day 5; late: treated on day 9), Experiment 2 (early: day 4; late: day 4), and Experiment 3 (early: day 7; late: day 11); c) PYN-treated mice from Experiment 1.2 (early: treated on day 4; late: treated on day 9), Experiment 2 (early: day 4; late: day 4), and Experiment 3 (early: day 7; late: day 11). Regression and statistical tests were performed using GraphPad Prism.*

We looked at all of our results to observe a correlation between initial parasitemia and clearance rate, represented by either 6- or 8-hour parasite reduction ratio (PRR). In Experiment 1 (Figures 2.9a-c) and Experiment 3 (Figures 2.9g-i), samples were taken at 6 hours following treatment, allowing calculation of a 6-hour PRR; in Experiment 2 (Figures 2.9d-f), the closest sample was taken at 8 hours. While there may be a small correlation between PRR and initial parasite count, it appears that the phase of growth is a far more important factor. We can conclude this because in most cases, even when late-treated groups exhibited a smaller PRR, they tend to cluster horizontally according to growth stage. This indicates that for each drug there is a PRR that is characteristic of the plateau phase of growth rather than a given starting parasite count. In the latter case, we would expect a stronger negative linear correlation as starting parasitemia increases.


a) 50mg/kg AS twice q24
6 hr PRR
Initial Parasitemia
b) 120mg/kg AQ twice q24 followed by 60mg/kg AQ on the third day
6 hr PRR
Initial Parasitemia
c) 60mg/kg PYN in a single dose
6 hr PRR
Initial Parasitemia
d) 50mg/kg AS three times q8
8 hr PRR
Initial Parasitemia
e) 50mg/kg AQ as a single dose
8 hr PRR
Initial Parasitemia
f) 10mg/kg PYN in a single dose
8 hr PRR
Initial Parasitemia

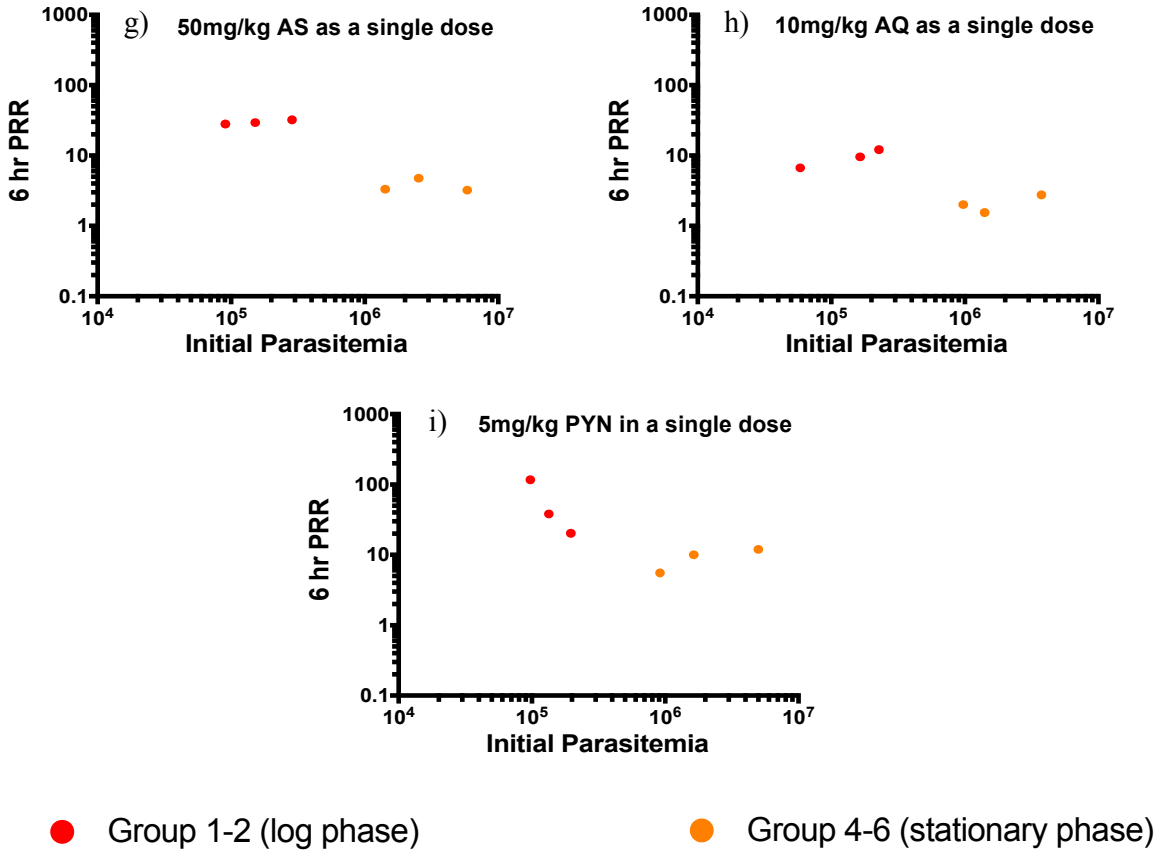
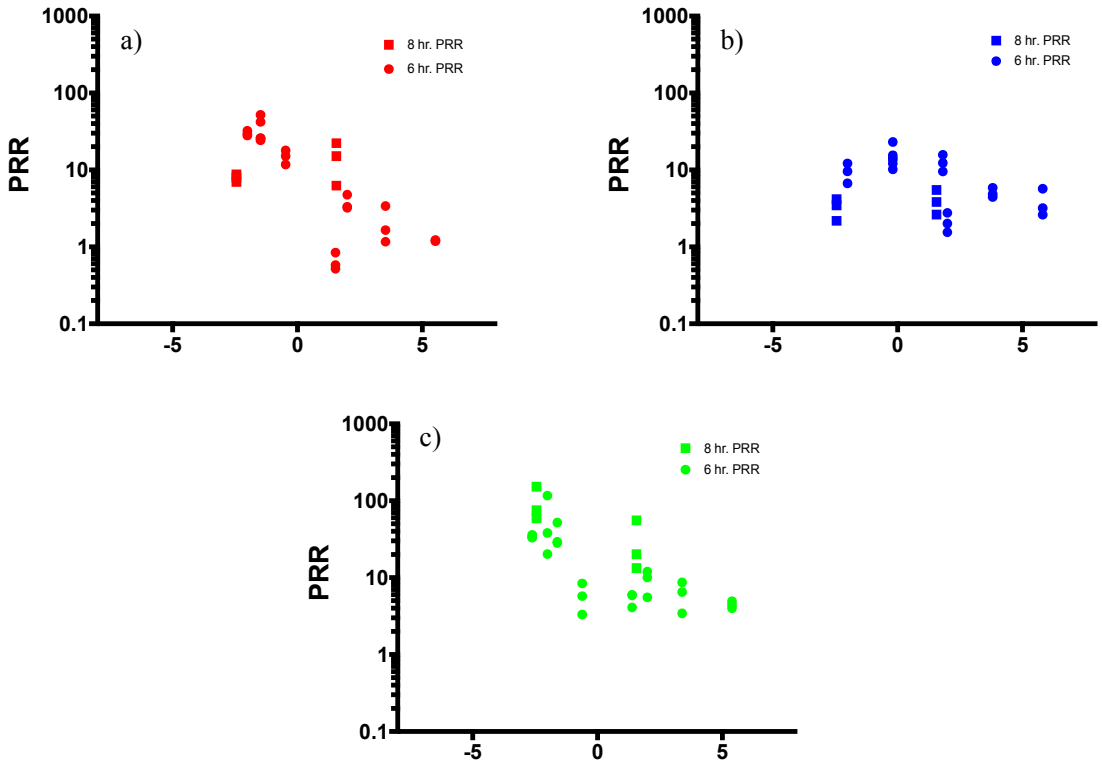


Figure 2.20 *Initial parasite count compared to early PRR (6- or 8-hours following treatment). a) Experiment 1.0, 50mg/kg AS twice q24; b) Experiment 1.1 120mg/kg AQ twice q24 followed by 60mg/kg AQ on the third day; c) Experiment 1.2, 60mg/kg PYN in a single dose; d) Experiment 2, 50mg/kg AS as a single dose; e) Experiment 2, 10mg/kg AQ as a single dose; f) Experiment 2 5mg/kg PYN as a single dose; g) Experiment 3, 50mg/kg AS as a single dose; h) Experiment 3, 10mg/kg AQ as a single dose; i) Experiment 3, 5mg/kg PYN as a single dose.*

Finally, we normalized treatment day to the center of the transition between log and plateau growth. Using the growth curves from the untreated mice in each group, we performed a non-linear regression with GraphPad to fit a quadratic polynomial to the region immediately surrounding the suspected inflection point. The point of inflection for the whole group was defined as the x-value (time) at which the apex of the parabola lied. For all three drugs, but most significantly for AS and PYN, there is a clear downward trend as treatment is started later in the course of infection (Figure 2.10).



Exp. number	1.0	1.1	1.2	2.0	2.1
Est. inflection point (day post-infection)	4.475	5.189	5.614	6.444	9.007

Figure 2.21 *PRR (either 6- or 8-hour ratio, depending on sampling pattern in the study arm) in mice treated at different points throughout the course of infection. In order to better compare study arms with disparate parasite growth phenotypes prior to treatment, the timing of the initiation of treatment was normalized to the point of transition between the log and plateau growth phases. a) all AS-treated mice, b) all AQ-treated mice, c) all PYN-treated mice.*

Discussion

We feel that our results are sufficient to suggest a relationship between the stage of growth of the *P. berghei* parasites and the kill rate immediately following treatment. Further, our results may suggest a continuous decline in kill rate as the infection progresses. This relationship could hold clinical relevance in that, while it is always preferable to quickly reduce parasitemia, rapid clearance of parasites is especially vital in cases of severe malaria, because most deaths occur in the first 24 hours. Therefore, it is

essential to understand any differences between the clearance kinetics in complicated, hyperparasitemic infections and uncomplicated infections so each can be optimally treated.

Few studies in humans have aimed to compare the treatment of hyperparasitemic malaria with cases of lower parasite density. One such study reported an increased instance of severe resistance to treatment among the hyperparasitemic patients (Sowunmi *et al.*, 2004). Our results were not in accordance with a traditional definition of resistance, as the outcome did not seem to be affected by the timing of the initiation of treatment. Nevertheless, the delay in parasite clearance observed in our studies could be associated with an increased risk of treatment failure. Importantly, however, our study did not recover a significant correlation between kill rate and parasitemia; instead, the duration of the infection prior to treatment seemed to be a better prognostic indicator. This relationship is even less well documented in human patients. A suitable proxy may be the distance patients must travel to reach professional clinics, as distance from a clinic is positively correlated with a delay in treatment seeking (Glik *et al.*, 1989; Ettling *et al.*, 1989; Kaewsonthi *et al.*, 1986). This relationship has not been studied from a pharmacodynamics standpoint.

Despite reaching statistical significance at several points throughout the first 2 days following treatment, the difference in clearance by early- and late-treated mice was sometimes unclear. While our data did not observe that late-treated mice clear parasites faster, there were some data that suggested, perhaps, that the clearance rates were the same. For PYN and AS, we assume that the lack of discrepancy between early- and late-treated mice can be attributed to the extraordinary rate of action of these drugs; their

characteristic clearance rates must approach the maximum splenic clearance rate even in late-treated mice, so early-treated mice cannot increase this rate greatly. We also observed much greater variation in the early clearance rates of late-treated mice, which could be both a phenotypic feature of treatment during the plateau phase and a reason for the ambiguity in the comparisons of some of our clearance curves.

The clearance curves of early- and late-treated mice, treated with any of the three drugs, are reminiscent of the delayed parasite clearance phenotype observed during artemisinin therapy in Southeast Asia– an increased parasite clearance half-life and clearance time, but little or no associated reduction in cure rate (Dondorp *et al.,* 2011). Mutations in the K13 gene, associated with this phenotype in *P. falciparum*, is unlikely to be the causative agent of our observations (Cao *et al.,* 2017). Nevertheless, Dogovksi *et al* (2015) hypothesized that K13 mutations are associated with enhanced antioxidant stress responses and explained that the mechanism of action of DHA, the bioactivated form of artemisinin and its derivatives, is similar to the traditional cell stress response. Mok *et al* (2014) hypothesized that the upregulation of the unfolded protein response as a result of K13 mutation allows for an increased capacity of the parasite to repair or remove proteins, counteracting the action of the drug. In our context, it is possible that quorum sensing activation initiates a similar cell stress response, giving the parasites a head start in their ability to repair damaged proteins and stave off death. Transcriptionally, this could be tested in the log phase of growth compared to the plateau phase.

Canonically speaking, this hypothesis is primarily applicable to artemisinin treatment, and therefore would only explain the delayed clearance in our AS trials.

However, the cell stress responses are also involved in the killing mechanism of the aminoquinolines, such as AQ and PYN, because they inhibit hemozoin formation. Free, non-detoxified heme leads to the formation of various reactive oxygen species (Francis *et al.,* 1997; Freinbicherler *et al.,* 2011), necessitating activation of the antioxidant response. Further, the immune response itself can alter the redox balance between the parasite and the host (Becker *et al* 2003). Dogovski *et al* (2015) showed synergy between DHA and epoximycin, a proteasome inhibitor; a similar experiment to see if the discrepancies shown in the present study are ameliorated would be worthwhile.

In addition to the changing milieu in the parasite and host as the infection progresses, the host preference of *P. berghei* shifts from normocytes to reticulocytes in late-stage infections, eventually reaching a preference for reticulocytes of about 150-fold over that of normocytes (Singer, 1954; Cromer *et al.,* 2006). The physiology of reticulocytes could contribute to the altered pharmacodynamics in late-treated mice. Srivastava *et al* (2015) hypothesize that the extensive metablome of reticulocytes provides resource reserves for *P. berghei* and *P. vivax* parasites (which exhibit preference for reticulocytes), potentially dampening the effect of the drugs on the parasites. In addition, human reticulocytes have greater antioxidant activity than normocytes, so the transition in host cell preference could also slow kill rates in much the same way as the parasite antioxidant response as hypothesized above (Sailaja *et al* 2003). If our observation that later initiation of treatment results in slower initial kill rates is dependent on an intrinsic characteristic of reticulocytes, then we may be unable to extrapolate to *P. falciparum*. *P. falciparum* also shows some preference for reticulocytes, but there is

some evidence to suggest that it becomes less selective at higher parasitemias (Simpson *et al.,* 1999).

We also thought it possible that the delay in maximum clearance observed particularly in the late-treated AS groups was due to altered pharmacokinetics. The pharmacokinetics of AS in severely ill human patients has been compared to that in those with uncomplicated infections, and while AS exhibited a slightly shorter half-life in severe infections, the half-life of DHA was not significantly affected (Li *et al.,* 2005). Similar studies are not available for AQ or PYN, but because all three drugs exhibited a correlation with progression of infection, the parsimonious explanation would be one in which all three are affected by the same mechanism. We therefore conclude that altered pharmacokinetics are not sufficient to cause such an altered clearance phenotype.

It is noteworthy that, in Experiment 1.0, the clearance action of AS is delayed for about 6 hours, well beyond the mean residence time for AS or DHA (Davis *et al.,* 2001). In the absence of altered pharmacokinetics, this is suggestive of an enhanced dependence on a delayed antibiotic effect, wherein damaged parasites either do not die or are not cleared for some time following exposure to drug.

Assuming these trends hold in *P. falciparum* infection, our results would again underscore the importance of early detection and treatment of malaria infection. In addition, this characterization of the parasite clearance curve, for these and other drugs that may be studied in the future, may help inform the ideal course of treatment in severe or hyperparasitemic malaria. Because clearance of *Plasmodium* parasites during chemotherapy appears to depend in some capacity on quorum sensing, our current

treatment regimens may be inadequately attuned to the changing physiology of the parasite or host as the infection progresses.

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Curriculum Vitae

Scott Meredith was born on September 21, 1995 in Baltimore, MD.

He started at Bucknell University in Lewisburg, PA in 2013 and was awarded a Bachelor of Science degree in Biology in 2016. While at Bucknell, he performed undergraduate research through the Howard Hughes Medical Institute expanding the bacteriophage library. He graduated *magna cum laude* in three years. Afterwards, he continued his education at the Johns Hopkins Bloomberg School of Public Health, studying for a Master of Science degree. Starting in 2016, he studied malaria chemotherapy in the lab of Dr. David Sullivan as part of the Johns Hopkins Malaria Research Institute.

In 2018, Scott will begin work in the lab of Dr. Sanjai Kumar at the Food and Drug Administration as an ORISE Fellow in the CBER Office of Blood Research and Review.